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THE UNIVERSITY OF ALBERTA

EFFECT OF RESERPINE ON THE REACTIVITY OF ISOLATED RABBIT BLOOD VESSELS

A Thesis
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

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by

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ABSTRACT

The effect of pre-treating rabbits with reserpine upon the reactivity of isolated aorta and carotid artery to sympathomimetic amines and electrical stimulation was studied. Estimates for the catechol amine concentrations of aorta and carotid artery from untreated and reserpine pre-treated rabbits were obtained using the tri-hydroxyindole fluorimetric method of assay.

In accord with Burn and Rand's hypothesis, after pre-treatment of the rabbit with reserpine, an increased sensitivity of aortic strips to sympathomimetic amines occurred concomitantly with a decrease in the concentration of catechol amines in the aorta. Reserpine pre-treatment also significantly lowered the concentration of catechol amines in carotid artery. The secondary response normally following cessation of application of an A.C. stimulus to isolated strips of aorta and carotid artery was reversibly inhibited by adrenergic blocking agents and could be abolished by pre-treating the animals with reserpine. The secondary response could not be restored in strips from reserpinised rabbits by treatment of such strips with sympathomimetic amines. It is suggested that this secondary response is due to liberation of noradrenaline from noradrenaline stores, postulated by Burn and Rand to be present in blood vessel walls.

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Unless otherwise indicated in the text, the names of drugs used in the work described in this thesis have been abbreviated as follows:

Adrenaline (A)	refers to	Adrenaline bitartrate
Noradrenaline (NA)	" "	Noradrenaline bitartrate
C. A.	" "	Catechol amine
Histamine	" "	Histamine acid phosphate
Dopamine	" "	3, 4-dihydroxyphenylethylamine hydrochloride
Dopa	" "	3, 4-dihydroxyphenylalanine
Acetylcholine	" "	Acetylcholine bromide
Tolazoline	" "	Tolazoline hydrochloride (Priscoline-Ciba)
Piperoxan	" "	Piperoxan hydrochloride (Benodaine-Merck, Sharp and Dohme)
Ilidar	" "	Azapetine phosphate (Ilidar-Roche)
Dibenzyline	" "	Phenoxybenzamine hydrochloride (Dibenzyline-Smith, Kline and French)
Bretylium	" "	Bretylium tosylate (Darenthin-Burroughs Wellcome)
Phentolamine	" "	Phentolamine Methane sulphonate (Rogitine-Ciba)

CHAPTER I

INTRODUCTION

contracted and on warming it slowly relaxes until at 37°C it reaches a steady level. Raising the temperature above 37°C produces a slow rise in tone.(13) The slow relaxation to a steady baseline can be hastened if the artery is placed under tension. The importance of application of a load to stretch the artery is discussed by Meyer (1906)(8), Macht (1914)(14), Rothlin (1920)(15), Ducret (1931)(16), Lewis and Koessler (1927)(14) and others.(18)(19)(20) Furchgott and Bhadrakom (1953)(9) attribute the gradual elongation of the isolated artery under a load to some physical process other than to a loss of tone, because the application of sodium nitrite, which is able to suppress completely low to moderate tone in aortic strips, did not alter the time characteristics of the elongation. Tone may sometimes be induced in a preparation by the application of stimulant drugs(21)(22) or by the addition of blood or serum to the bathing solution.(8)

Frequently associated with the development of tone in isolated blood vessels is the development of spontaneous contractions. The mechanism whereby spontaneous contractions are produced is unknown, although temperature(16), tension(10), degree of oxygenation(18), duration of storage of the blood vessel after its removal from the animal(10) and ionic environment(18) are factors which influence the frequency and intensity of these contractions.

Effect of Temperature and Load

The response of isolated vascular preparations to drugs is maximal at 37°C.(8)(16) If the load applied to the artery exceeds a critical level, the reactivity to stimulant drugs is reduced.(16)

Bathing Medium

The ionic content of the solution in which isolated arteries are suspended governs their degree of reactivity to drugs. Meyer in 1906 used Ringer's solution, as did other early workers.(23)(24)(25)(19)(16)(26)(18) Meyer found that the reactivity of his preparation deteriorated in tap water and if 0.5% potassium chloride was added to the Ringer's solution. Apitz (1920)(18) and Schmidt (1921)(25) studied the effect of increasing the concentration of salts in Ringer's solution and of adding magnesium to Ringer's solution. Locke's solution was used by later workers(14)(20)(17) and following the work of Krebs(27) in 1933, Krebs-Henseleit and Krebs-bicarbonate solution were introduced as bathing media suitable for isolated blood vessels. The Krebs solutions contain phosphate in addition to bicarbonate and thus better buffered than are Locke's or Ringer's solutions (Table 1).

Effect of Oxygenation

Oxygenation of the bathing medium is not essential for the production of responses of the isolated blood vessels to stimulant drugs and to electrical stimulation(8)(28) but it increases the height of contraction and the speed of recovery(22)(8) of such responses. For experiments of long duration, oxygenation favours maintenance of the responsiveness of the blood vessel to stimulant drugs, although it may tend to increase or elevate tone in the preparation. (8)

Effect of pH

Furchgott(9) recommended that the pH of the bathing medium be adjusted to 7.4, which is the pH obtained if a mixture of 95 percent

TABLE 1

COMPOSITION OF PHYSIOLOGICAL SALINES

IN GM. PER LITRE

	FROG RINGER	LOCKE	KREBS HENSELEIT
Sodium Chloride	6.5	9.0	6.92
Potassium Chloride	0.14	0.42	0.35
Calcium Chloride (anhydrous)	0.12	0.24	0.29
Potassium dihydrogen phosphate	-	-	0.16
Magnesium sulphate (hepta hydrate)	-	-	0.29
Sodium bicarbonate	0.2	0.5	0.19
Glucose	2.0	1.0	2.0

oxygen and 5 percent carbon dioxide is bubbled through Krebs-bicarbonate. He confirmed the reports of Apitz(18) and Schmidt(25). that the reactivity of isolated blood vessels is reduced in acid solution.

Metabolic Effects

Studies on the oxygen consumption of isolated arteries by the Warburg method(29)(86)(87)(49)(88) report a QO_2 considerably lower than that for cardiac and skeletal muscle, but of the same order of magnitude as that for smooth muscle isolated from the rabbit small intestine.(22) Briggs(29) and Furchgott(22) have found that pyruvate, acetate, butyrate and succinate as well as glucose, can act as energy yielding substrates for arterial smooth muscle. Furchgott concluded from these and other results, that there is a glycolytic and a fatty acid oxidative system in vascular smooth muscle, both supplying "active acetate" to the Krebs cycle.

A useful property of isolated arteries, which was noted by Meyer, is that of retaining their excitability, if stored at 2 - 3°C, for periods of 3 to 14 days.(8)(14)(17)(30) The susceptibility to adrenaline is conditioned by the temperature of storage(31) and decreases as the duration of storage increases.(16)

Efferent Innervation

Both aorta and carotid artery are innervated by sympathetic vasconstrictor fibres, but the former is activated by excitation of these fibres to a greater extent than is the latter.(32) The innervation of aorta and arteries is very rich and plexuses are found within the various tunicas of the blood vessel wall.(33) Large numbers of these nerve

fibres still persist after sympathectomy(33), so that it can be expected that isolated blood vessels will contain appreciable numbers of sympathetic nerve endings.

Orientation of Smooth Muscle

Methods of preparing isolated blood vessels for in vitro use have changed from the narrow rings advocated by Meyer to the spirally cut strips first described by Lewis and Koessler (1927).(17) This latter technique has been elaborated by Furchgott and Bhadrakom(9) using isolated rabbit aorta. Furchgott(22) stressed the importance of the orientation of the smooth muscle fibres when considering the reaction of isolated arteries or strips of arteries to drugs. The spiral method of cutting the arterial strip was instituted because there is little or no longitudinal smooth muscle in most arteries.(22) It is possible, however, that the circular smooth muscle is orientated at such an angle that strips cut in left handed spirals show a reactivity considerably different from strips cut in right handed spirals. Lum and Rashleigh (1961)(84) found that with cat carotid artery, strips cut counter clockwise responded to noradrenaline whereas strips cut clockwise showed very little or no response to noradrenaline.

Species Differences

Isolated arteries from different species, even when prepared in exactly the same way, show marked differences in drug sensitivity. Spontaneous activity tends to occur with arteries from certain species, making such arteries unsuitable for testing the effects of drugs (page 2). (19)(18)(16) The carotid artery is the artery generally used and it

possesses an advantage over the aorta in having a higher proportion of smooth muscle.(34) In smaller animals, however, it is rather difficult to cut spiral strips from the carotid artery and therefore the aorta, being larger, is used.

RESPONSE OF ISOLATED BLOOD VESSELS TO ELECTRICAL STIMULATION AND TO DRUGS

Response to Electrical Stimulation

Meyer(8) and Kosuge (1934)(35) obtained contraction of isolated arteries on the application of a high inductance discharge. Both found that there was a latency period of at least one second and that the contraction was slow to return to the baseline. Using cocainised preparations of the retrolingual membrane of the frog, Fulton and Lutz (1942)(36) obtained constriction of the arterioles by direct stimulation with microelectrodes through an induction coil. To produce an effect with direct current, smooth muscle requires voltages which are much higher than those required by skeletal or cardiac muscle.(37) The arterial strip cannot be stimulated by square pulses of low voltage and short duration, and if the voltage is increased, there appears to be some damage to the strip, because the response to other types of stimuli is reduced.(38) However, as with many other smooth muscle preparations, arterial strips respond more vigorously to short periods (5 to 10 seconds) of alternating current stimulation than they do to single shocks.(22) Furchgott(28) found that application of an alternating current to spirally cut rabbit aortic strips produced a rapid contraction during current flow, followed by a slower contraction and prolonged relaxation on cessation of the current. The contraction after current flow could

be abolished by dibenamine, suggesting that this secondary contraction was due to liberation of catechol amines from the aorta wall. Schmiterlow (1948)(40) and Euler and Lishajko (1958)(41) demonstrated that the arterial wall does contain noradrenaline.

Response to Drugs

Isolated blood vessels are particularly sensitive to the stimulant actions of adrenaline and noradrenaline(9)(42) and in addition contract to 5-hydroxytryptamine(43), histamine(22), acetylcholine(22) and nicotine.(44) As a rule these preparations are incapable of showing a depressor response but, if some tone is induced by the addition of a stimulant drug such as adrenaline or histamine, a depressor response to pitressin(22) or dopamine(45) can be obtained.

Very little is known about the influence of in vivo treatment of animals with drugs upon the reactivity of in vitro preparations from such animals. In addition, results obtained from in vitro preparations can be applied only with reservation to the same tissues in the intact animal. Nevertheless, some indication of the peripheral effects of drugs can be obtained using this technique and with in vitro preparations it is possible to control the many variables which make investigation of the peripheral effects of a drug in the intact animal, very confusing. A drug which has been examined in this way is the alkaloid reserpine.

ACTIONS OF RESERPINE ON VASCULAR SMOOTH MUSCLE

Reserpine, a drug used for many centuries in India in the treatment of a variety of conditions ranging from mania to snakebite,

has in recent years been introduced into western medicine. Although its effectiveness in lowering the blood pressure, particularly in those cases in which hypertension is caused or aggravated by anxiety, is unquestionable, its mode of action is still in dispute. It acts both centrally and peripherally to reduce sympathetic tone, probably by a different mechanism at each site. Direct effects on the metabolism of isolated vascular tissue have been demonstrated. Reserpine apparently interferes with the carbohydrate metabolism with a resultant decrease in availability of energy for muscular contraction in smooth muscle. (46)(47)(48) Chronic administration of reserpine to rats resulted in aortic QO_2 values which were significantly lower than those obtained with untreated animals.(49) Thus an interference with oxidative metabolism may explain the reduction of inherent tone in vascular smooth muscle following treatment with reserpine.(30)

The loss in peripheral vascular tone following reserpine may also be the result of a depletion of catechol amines associated with the sympathetic nerve endings of the tissue. This depletion can occur in adrenal glands(50), sympathetic nerves(52), heart(53), brain(56)(54), nictitating membrane(55) and aorta(51) following pre-treatment of the animal with reserpine. Reserpine acts slowly, a latency period being noticeable both in its action to lower the blood pressure and to deplete organs of catechol amines.(53)

Kirpekar and Lewis (30)(57) found that reserpine added to the organ bath antagonised the contractile responses of all spasmogens tested, upon strips of carotid artery from normal horses. This antagonism was, however, frequently irreversible. The only reports on

the effect of reserpine pre-treatment on the reactivity of in vitro preparations of blood vessesl are those of Burn and Rand(51) and Gillis and Yates.(58) Burn and Rand found that aortic strips from rabbits pre-treated with reserpine were more sensitive to noradrenaline than were similar strips obtained from untreated animals. Gillis and Yates found that pre-treatment of rabbits with reserpine had the same effect as dibenamine in abolishing the secondary contraction which normally follows cessation of application of an A.C. stimulus. This observation, together with the observation by Burn and Rand that reserpine decreases the concentration of catechol amines in the aorta, suggests that the secondary contraction seen with application of an A.C. stimulus is due to catechol amine release. Burn and Rand's observations of the effect of pre-treatment with reserpine on in vitro preparations of blood vessels agree very well with the effect reserpine has been shown to have on the vascular system in vivo. On a variety of preparations, pre-treatment with reserpine has been shown to lead to an increased sensitivity to tyramine.(59)(60)(55)(61)(62) Denervation of an organ has the same effect(63)(64)(65) and it was in explanation of this action of denervation that Burn first proposed his "adrenaline stores" hypothesis in 1932.(63)

BURN AND RAND'S HYPOTHESIS

According to this hypothesis, there is, at or near the end of the sympathetic nerve in the blood vessel, a store of catechol amine. Sympathomimetic amines are postulated to be of two types, those like noradrenaline which act directly on the receptors and those like tyramine

which release noradrenaline from these stores; this released noradrenaline being responsible for the action of tyramine. Both reserpine and denervation have been shown to lead to a fall in the organ content of noradrenaline and at the same time to abolish the action of amines such as tyramine. Under normal conditions, it is postulated that noradrenaline is continuously being released from and taken up by the stores and that this released noradrenaline, acting upon the adrenergic receptors, is responsible for the maintenance of vascular tone. The increased sensitivity to noradrenaline after reserpine or denervation is due to there being more free receptors with which the exogenous noradrenaline can react, since the amount of endogenous noradrenaline has been reduced. Evidence that tyramine acts in the untreated animal by release of noradrenaline is afforded by the work of Lockett and Eakins(66) who found that concentrations of adrenaline and noradrenaline in plasma were markedly increased during pressor responses to intravenous tyramine in cats. If the mechanism of action of reserpine is to liberate amines from the stores, it should be possible to antagonise this action, as judged by the production of a response to tyramine in a reserpinised animal, by infusion of noradrenaline. This has been proved possible using rat blood pressure(67), cat blood pressure (68) and cat nictitating membrane.(68) However, an increase in the concentration of tissue catechol amines following such an infusion has not been demonstrated, even although the response to tyramine is restored.(69)(67) Pennefather and Rand(69) found that, in spinal cats, the uptake of noradrenaline from an infusion was less in the reserpinised

than in the untreated animal, suggesting an interference with the noradrenaline binding mechanism by reserpine.

STATEMENT OF THE PROBLEM

In the work to be described, an attempt has been made to examine generally the influence of reserpine pre-treatment on the reactivity of isolated vascular tissue to a variety of cardio-active drugs and also to electrical stimulation. In so doing, the applicability of the Burn and Rand hypothesis to an isolated system has been studied.

Footnote

Definition of "tone" as used in this chapter:

The ability of isolated blood vessels to relax in response to heat, stretch, oxygen or drugs has been taken to indicate that such blood vessels possess "tone".

CHAPTER II

ASSAY OF CATECHOL AMINES IN EXTRACTS OF AORTA AND CAROTID ARTERY

INTRODUCTION

Catechol amines may be estimated chemically or pharmacologically. The property of fatigue inherent in biological preparations, make it difficult to perform quickly as many biological assays as can be done using chemical methods of estimation. Also, pharmacological methods, in addition to taking longer to master, are more time-consuming to perform than are chemical methods. Chemical methods, not being subject to biological variation, are more reproducible. For these reasons, chemical methods are to be favoured, particularly for the detection of such low concentrations of the amines as are found in blood vessels. Although usually more sensitive, the specificity of chemical as compared with pharmacological methods may sometimes be questionable.

Almost all the blood vessel extracts were assayed chemically, but a few were assayed biologically (page 20).

METHODS

Extraction of Catechol Amines from Aorta and Carotid Artery of the Rabbit

Only one to two hundred milligrams of thoracic aorta can be obtained from one rabbit and assuming a normal noradrenaline content of about 0.47 μg/gm.(51) it is clear that in order to obtain a measurable concentration of noradrenaline the volume of the extract solution must be kept as low as possible. Purification of the extract solution is desirable, but, depending on the specificity of the method of assay, may not be essential. If the volume of the extract solution is very low, purification may not be possible.

Two methods for the extraction of aorta were used; one which included a purification technique and one which did not. The latter was found to be more satisfactory and was used for almost all the estimations. However, for the sake of completeness, a description of the former is included.

The preparation of the solutions used in the extraction procedure is described in Appendix 1.

(i) Extraction of Aorta and Purification of the Extract Solution

The tissue, immediately after removal from the animal, was quickly washed free of blood using normal saline, dried on filter paper and weighed on a torsion balance. After weighing, it was dropped into about 6 mls. of a 1:1 mixture of 10% trichloroacetic acid and normal saline, and homogenised in a glass homogeniser in an ice-bath. The residue was spun down at 3000 r.p.m. for about 5 minutes, the supernatant decanted and adjusted to 10 mls. with 5% trichloroacetic acid.(70)

This acid solution contains catechol amines and any other basic materials which may be present in the vascular walls. Schmiterlöw(40) mentions that histamine and acetylcholine are present in acid extracts of cow aorta. Isolation of the catechol amines can be achieved by adsorbing them onto alumina under alkaline conditions.(71) However, this method has the disadvantage that not only adrenaline and noradrenaline are adsorbed, but also any other di-enols which may be present in the solution.(72) The only di-enol likely to be present in vascular walls is dopamine. If the extract is assayed biologically, dopamine will scarcely interfere as it has only one-fiftieth to one-hundredth the pharmacological activity of noradrenaline.(73) If the

extract is assayed fluorimetrically, interference by dopamine can be excluded by choosing a method of assay which will produce fluorescent derivatives of adrenaline and noradrenaline but not of dopamine.(74) The catechol amines may be adsorbed by one of two methods viz:

(a) Adsorption onto alumina in a chromatographic column: This is the method used by Lund and Cohen(72) who obtained recoveries from such columns of 70% and 90% respectively.

(b) Adsorption onto alumina using the batch method of Drujan, Sourkes et al(75): This was the method followed in this work, principally because it is easier to set up and run a succession of samples by this method than by method (a).

Cationic resins, for example Dowex 50, have been used with considerable success in the isolation of catechol amines from tissue extracts(76)(77). Such resins possess the advantage over alumina that they will adsorb the amines at an acid pH, an alkaline pH being required for successful adsorption on alumina. Bertler et al(77), using a Dowex 50 resin in a column, quotes recoveries of nearly 100%; this high recovery is probably due to the greater stability of catechol amines at an acid pH.

Purification of the extract solution using the method of Drujan, Sourkes et al(75): The amines were adsorbed onto alumina under alkaline conditions and eluted from the alumina using 0.1N hydrochloric acid, as described by Drujan, Sourkes et al. The acid eluate may be kept in a refrigerator for at least a week without loss of catechol amines.(40)(78)(77)

(ii) Extraction of Aorta or Carotid Artery with no Purification Technique

After removal from the animal, the thoracic aorta was cut spirally and the lower part assayed, the upper 3 cms. being reserved for pharmacological tests in an organ bath. The aortas to be assayed were suspended for 45 minutes in oxygenated Krebs-Henseleit solution at 37°C under a tension of 2 gm. After this time they were removed from the organ bath, quickly trimmed of adhering fatty tissue and blotted dry on filter paper and dropped into a flask of liquid nitrogen. Tissues could be kept thus frozen solid in a deep freeze refrigerator for several days until extraction.

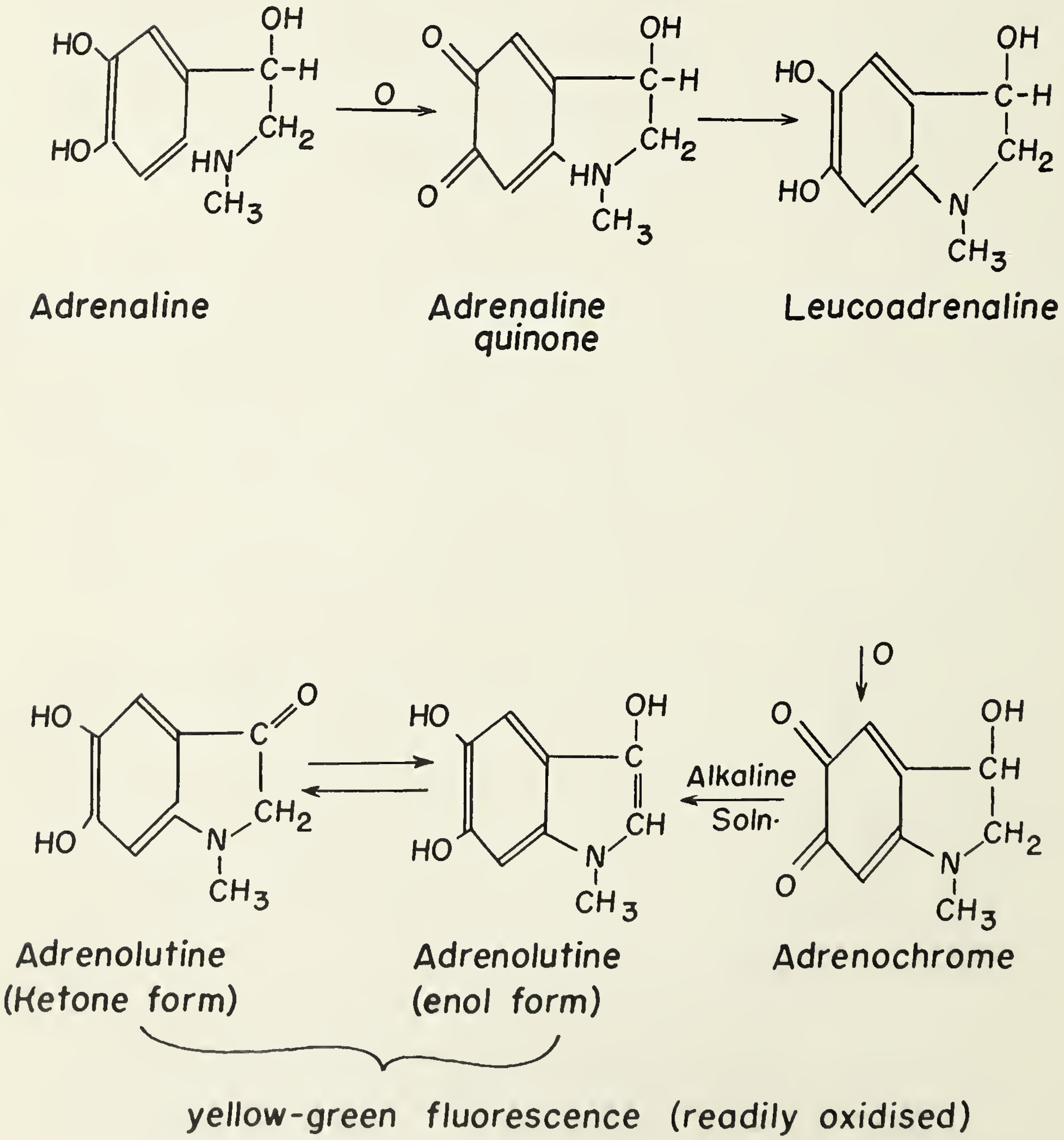
Some experiments to test the effect of electrical stimulation on the content of catechol amines in rabbit aorta were carried out. The lower part of the aorta was treated as described above and the upper part stimulated electrically with 15 volts for 10 seconds every 12.5 minutes for 6 to 10 hours, before being removed, blotted dry and frozen in liquid nitrogen.

The carotid arteries were not suspended in Krebs-Henseleit, but were dropped into liquid nitrogen immediately after removal from the animal. Two arteries, one from each of two rabbits, were pooled for each assay. This was found to be necessary due to the low weight of a single rabbit carotid artery.

For extraction, the frozen tissue was removed to a cold room at 4 - 8°C where it was pulverised, using a chilled metal test-tube with fitted metal plunger after the method described by Furchgott and Gubareff.(79) The powdered tissue was weighed on a torsion balance and

Figure I.

Chemical Constitution of Adrenolutine.



extracted for 20 minutes with 2 mls. 0.3N perchloric acid using a magnetic stirrer, in the cold room. The suspension thus obtained was spun at 3000 r.p.m. for about 5 minutes to obtain a clear supernatant which was removed by Pasteur pipette into a small beaker and frozen solid on dry ice. This acid extract was stored in the frozen state until required for assay.

Extraction procedure (ii) was superior to procedure (i) because the entire operation was carried out in the cold room. In (i), while every precaution was taken to keep the extract solutions chilled in ice, the procedure was carried out at room temperature, thus making temperature control of the various steps difficult.

Estimation of Catechol Amines in Vascular Extracts Using the Tri-hydroxyindole Fluorimetric Method.

The method of Lund(71) and Euler and Floding(80), with modifications in the differential assay of adrenaline and noradrenaline introduced by Price and Price(81) and Cohen and Goldenberg(72), was followed. Adrenaline and noradrenaline can be converted to their fluorescent lutine derivatives by oxidation, followed by the addition of strong alkali under oxygen free conditions. The fluorescent derivative is probably the 1-Me-5-6-dihydroxy derivative of indoxyl(82), which has itself a strong yellow fluorescence and is the nucleus of a number of fluorescent compounds (fig. 1).

The fluorescent derivatives of adrenaline and noradrenaline differ sufficiently in their activation spectra, to allow a differential estimation of the two amines by measuring the fluorescence intensity at two different activation wavelengths.(77)(72)(81) The activation and

fluorescence wavelengths recommended by different authors vary, probably due to slight differences in technique and also to variation among instruments.(72)(71)(81) Linear relationships were obtained between percent fluorescence and concentration from 0.005 μg/ml. to 0.05 μg/ml. noradrenaline and adrenaline respectively, at activations of 400 mμ and 445 mμ. These activation wavelengths were chosen because the ratios of adrenolutine to noradrenolutine fluorescence at these wavelengths differed appreciably, being 1:1 and 3:1 respectively.

In assaying an extract for catechol amines, the amines may be expressed as adrenaline and noradrenaline as outlined, or they may be assayed and expressed as noradrenaline. The latter procedure is frequently carried out for extracts containing low concentrations of the amines. Both methods of expression were used in this work. (Appendix II).

The Aminco-Bowman spectrophotofluorometer was used in the estimations. Preliminary experiments proved the Fisher nephotofluorometer to be less satisfactory, particularly with regard to ease of operation and use in the differential assay of adrenaline and noradrenaline.

The preparation of reagents used in this method of assay is given in Appendix I.

Treatment of the Extracts Prior to the Production of Fluorescence

The extracts prepared by extraction method (ii) (page 15) were all assayed fluorimetrically. The extracts were thawed and adjusted to pH 5 to 6 using a pH meter, by the stepwise addition of 0.8N potassium carbonate, with constant stirring on a magnetic stirrer. The volume of potassium carbonate necessary was noted. The precipitate

of potassium perchlorate was spun down by centrifugation at 3000 r.p.m. for 5 minutes, and the supernatant removed by Pasteur pipette.

Procedure of Assay

The lutines were developed by the method of Euler and Floding(80). Fluorescence was measured at 400 mμ and 445 mμ activation wavelengths. The fluorescence wavelength was set to 510 mμ for both readings. All wavelengths quoted are uncorrected instrumental values.

1.0 ml. of the supernatant obtained from neutralisation of the extract or 1.0 ml. of the standard adrenaline or noradrenaline solution was pipetted into a 10 ml. volumetric flask and the following steps carried out:

(a) 1.0 ml. 0.1M phosphate buffer pH 6.5 added

(b) 1.0 ml. 0.25% zinc sulphate added. (Bertler, Carlsson and Rosengren(77) found that zinc sulphate appeared to improve the agreement between internal and pure standards.)

(c) 0.1 ml. 0.25% potassium ferricyanide added to oxidise the amines to their chrome derivatives.

(d) 2 minutes after addition of the oxidant, 1.0 ml of a freshly prepared mixture of 9 parts 20% sodium hydroxide and 1 part 2% ascorbic acid was added. The sodium hydroxide rearranges the chrome to the fluorescent lutine and the ascorbic acid ensures that oxidation does not proceed further than the lutine.

(e) The solution was adjusted to 10 mls. using distilled water and mixed by inverting the volumetric flask several times.

(f) The samples were read at a fixed time of 10.5 minutes after

addition of the alkali. It is important that this time be kept constant, as the fluorescence varies with time.

Blanks

Extract (or in the case of the standards, reagent) blanks were prepared by adding only the alkali at 2 minutes, and allowing the fluorescence to decay for 5 minutes before adding the ascorbic acid.(71) The reading for the blank was subtracted from the reading for the sample to obtain the percent fluorescence due to the lutines alone. As with the samples, the fluorescence of the blanks varies with time and it is essential that exactly the same time is allowed for decay of the fluorescence of extract and reagent blank. Also the percent fluorescence for reagent and sample blank should be read at exactly the same time after the alkali has been added.

Standards

The standard solutions used were noradrenaline and adrenaline bitartrate (1 μg/ml.) dissolved in distilled water and adjusted to pH 3.5 to 4 with 0.1N hydrochloric acid using a pH meter. Fifty or 100 mls. of these solutions were prepared at one time. Stored at this acid pH and at 4°C, no deterioration could be detected for as long as the solutions were used (about 4 to 6 weeks). Each new set of standard solutions was compared with the previous set and with freshly prepared dilutions of the amines to check the accuracy of dilution. Prior to the start of the assays, values for percent fluorescence of adrenolutine and noradrenolutine at 400 mμ and 445 mμ activation wavelengths were obtained. When readings obtained from two different sets of standard solutions agreed closely

with one another and with freshly prepared dilutions of the amines, these readings were averaged. These average values were used in the calculation of adrenaline and noradrenaline in the extracts. Adrenaline and noradrenaline standards were run in parallel with each batch of extracts assayed to check that there was no change in the machine over a period of time. Usually there was very little difference between these standards and the average values and in such cases the average values were used for calculation, on the premise that averages from several different dilutions are more accurate than readings from one dilution. Occasionally there was a significant difference between the standards run with a given batch, and the averaged standards. In these cases, a series of standards were run and new averages taken.

Experiments Designed to Assess the Sensitivity and Accuracy of the Extraction Method and the Tri-hydroxyindole Method for Assay of Adrenaline and Noradrenaline

See Appendix III

Biological Estimation of Catechol Amines in Aortic Extracts

Isolated aorta strips from normal and reserpine pre-treated rabbits were set up as described later in this thesis (page 28). This assay depended upon a comparison of the magnitude of contraction produced by standard noradrenaline and aliquots of extract. An internal standard was prepared with each batch of extracts, by taking 200 ng noradrenaline through the entire extraction procedure. The pH of an aliquot of extract, before its addition to the organ bath, was raised to 6 (bromothymol blue) using 0.2N sodium hydroxide. This neutralisation

procedure was carried out immediately before adding each aliquot of sample to the organ bath, in order to minimise loss of catechol amines in the sample by prolonged exposure to a neutral or alkaline pH. Ten mls. of 5% trichloroacetic acid taken through the extraction procedure and added to the organ bath after being treated in exactly the same way as the samples had no effect on the test tissue.

Dilutions of noradrenaline were prepared every day in fresh Krebs-Henseleit solution from a stock noradrenaline solution (Appendix I) and were kept away from bright light.

The aortic strips were allowed to relax for about 2 hours after removal from the animal, or until they maintained a steady level of tone. Noradrenaline was then added in doses sufficient to give sub-maximal responses until the sensitivity of the preparation stopped increasing, which frequently took several hours. The samples and the noradrenaline were added to the organ bath, in random order, by tuberculin syringe using a number 24 needle, in volumes of 0.1 ml. or 0.2 ml. The drug or sample was washed out when the increase in tension of the aorta had ceased. A log dose-response line to noradrenaline was plotted and the catechol amine content of the samples, expressed as noradrenaline, was estimated by equating the observed response to the known volume of sample with a concentration of noradrenaline producing the same degree of contraction.

Percent recoveries of noradrenaline were calculated from the internal standards and used to correct for the loss of noradrenaline from the samples caused by the method of extraction and assay.

RESULTS

Methods of calculation and formulae used in calculation are given in Appendix II.

Estimation of Catechol Amines in Vascular Extracts Using the Tri-hydroxyindole Fluorimetric Method

Estimations of the recovery of amines added to segments of aorta and taken through the extraction and fluorimetric procedures were obtained (see Table 10 in Appendix III) but no correction for loss was applied to the values obtained from the extracts. It was decided that such correction was unnecessary as the aim of the assays was to reveal possible differences in concentrations from reserpinised and untreated animals, which would be seen whether or not a correction were applied.

Only about 0.03 μg total catechol amines per 1 ml. sample can be expected in an aortic extract and since this amount is made up largely of noradrenaline(40), the amount of adrenaline present must be well below 0.01 μg, which was found to be the lower limit for accurate assay of adrenaline in an adrenaline-noradrenaline mixture (Appendix III). This, together with biological variation, could explain the wide variation in the adrenaline: noradrenaline ratio encountered even in rabbits subjected to the same treatment. This ratio varied so widely between rabbits within treatments that it was impossible to come to any conclusion regarding the effect of reserpine to deplete vascular tissue of one amine rather than the other.

Despite the fact that the expected amount of catechol amines

TABLE 3

CATECHOL AMINES (expressed as NA μg base/gm) OF AORTA FROM UNTREATED AND RESERPINE PRE-TREATED (2 mg/kg 1/v) RABBITS, WITH AND WITHOUT ELECTRICAL STIMULATION, ASSAYED FLUORIMETRICALLY

Rabbit Number	Duration of Treatment	A + NA in Terms of NA	
		Without E.S.	After E.S.
1	Untreated	1.58	1.71
2		1.81	2.14
3		0.26	0.3
4	24 hours	0.33	1.32
5		0.40	0.32
6		0.64	0.08
7	4 hours	0.07	0.14
8		0.14	0.1
9		0.09	0.15
10	2 hours	0.11	0.1
11		0.13	0.17
Means		0.51	0.59
S.E.		±0.15	±0.18

TABLE 2

CONCENTRATIONS OF NORADRENALINE AND ADRENALINE (as μg base/gm) OF AORTA FROM UNTREATED AND RESERPINE PRE-TREATED (2 mg/kg l/v) RABBITS ASSAYED FLUORIMETRICALLY

Rabbit Number	Duration of Treatment	NA	A	NA + A in Terms of NA	Mean (NA + A), in Terms of NA ± S. E.	Significance P
1	Untreated	0.54	0.91	1.58	0.86 ± 0.12	
2		1.67	0.19	1.8		
3		0.2	0.22	0.4		
4		0.04	0.23	0.26		
5		0.32	0.39	0.68		
6		0.26	0.32	0.56		
7		0.34	0.28	0.60		
8		0.2	0.89	1.09		
9		0.47	0.69	1.16		
10		0.33	0.44	0.77		
11		0.6	0.06	0.66		
12		0.76	-ve	0.47		
13		0.27	1.12	1.39		
14		0.14	0.52	0.66		
15	24 hours	0.07	0.28	0.33	0.56 ± 0.08	<0.05
16		0.19	0.23	0.40		
17		0.26	0.41	0.64		
18		0.52	0.36	0.88		
19		0.44	0.19	0.63		
20		0.24	0.16	0.4		
21		0.22	0.16	0.39		
22		0.83	-ve	0.84		
23	4 hours	-ve	0.27	0.07	0.27 ± 0.12	<0.01
24		0.03	0.11	0.14		
25		0.04	0.05	0.09		
26		0.34	0.12	0.45		
27		0.52	0.06	0.62		
28	2 hours	-ve	0.13	0.11	0.35 ± 0.14	<0.01
29		0.18	-ve	0.13		
30		0.46	0.2	0.64		
31		0.14	0.03	0.17		
32		0.43	0.1	0.57		
33		0.61	-ve	0.5		

(Explanation of negative values is given in Appendix II) per extract sample is about 0.03 μg (which is only just above the lower limit of the assay method (Appendix III)), reasonably consistent results were obtained by assaying and expressing the total catechol amines in the extracts as noradrenaline. All statistical calculations were based on values obtained by this method.

Effect of Pre-dosage With Reserpine Upon the Concentration of Catechol Amines, Expressed as Noradrenaline in Aorta

The results (Table 2) were analysed using the analysis of variance to test for significance of difference between treatments. Duncan's multiple range test was used to test which two treatments differed significantly.

There was a significant difference in noradrenaline concentration between aortas from untreated and treated rabbits ($P < 0.01$), regardless of the duration of reserpine pre-treatment. There was no significant difference between aortas subjected to different durations of reserpine pre-treatment.

Effect of Electrical Stimulation upon the Concentration of Catechol Amines, Expressed as Noradrenaline, in Aorta

Using the t-test, no significant difference was found between the concentration of amines in aorta before and after electrical stimulation (Table 3). Aortas from treated and untreated rabbits were stimulated electrically and their catechol amine content estimated. From inspection of this data, reserpine pre-treatment appeared to have no influence on the change in catechol amine content of aorta caused by electrical stimulation. Therefore, for the purpose of calculating t,

TABLE 5

CATECHOL AMINES (expressed as NA μg base/gm) OF AORTA FROM UNTREATED AND RESERPINE PRE-TREATED RABBITS, ASSAYED BIOLOGICALLY

Rabbit Number	State of Rabbit	Recovery from Internal Standards	NA (Corrected for Recovery)	Mean NA ±S.E.
1	Untreated	17.5%	0.51	
2	"	30%	0.42	0.54±0.08
3	"	17.5%	0.69	
4	Reserpine 2 mg/kg 1/p 2 days before use Reserpine 2 mg/kg 1/v 1 day before use	21.7%	0.61	0.48±0.13
5	"	27.2%	0.35	

TABLE 4

CONCENTRATIONS OF NORADRENALINE AND ADRENALINE (as μg base/gm) OF CAROTID ARTERY FROM UNTREATED AND RESERPINE PRE-TREATED (2 mg/kg 1/v) RABBITS ASSAYED FLUORIMETRICALLY

Rabbit Number	Duration of Treatment	NA	A	NA + A in Terms of NA	Mean (NA + A), in Terms of NA ± S. E.	Significance P
1	Untreated	0.6	0.63	1.19		
2		1.18	0.85	2.02		
3		1.69	0.48	2.18	1.88 ± 0.19	
4		1.07	1.12	2.2		
5		1.19	0.49	1.83		
6	24 hours	0.07	0.07	0.14		
7		Blank > Sample no estimates		0.36		
8		Blank>Sample no estimates		0.87	0.48 ± 0.15	< 0.01
9		0.62	0.1	0.78		
10		0.27	-ve	0.25		
11	4 hours	-ve	0.34	0.32		
12	and	0.08	0.08	0.16		
13	2 hours	0.09	0.22	0.3	0.50 ± 0.16	< 0.01
14		0.64	0.42	1.0		
15		0.13	0.19	0.31		
16		0.30	0.43	0.92		

(Explanation of negative values is given in Appendix II) the amine concentrations in treated and untreated aortas before electrical stimulation were pooled and compared with the pooled concentrations in treated and untreated aortas after electrical stimulation.

Effect of Pre-dosage with Reserpine Upon the Concentration of Catechol Amines, Expressed as Noradrenaline, in Carotid Artery

The results (Table 4) were analysed using the analysis of variance to test for significance of difference between treatments. Duncan's multiple range test was used to test which two treatments differed significantly.

There was a significant difference in noradrenaline concentration between arteries from untreated and treated rabbits ($P < 0.01$), regardless of the duration of reserpine pre-treatment. There was no significant difference between arteries from animals subjected to different durations of reserpine pre-treatment.

Biological Estimation of Catechol Amines in Aortic Extracts

Using the t-test, there was no significant difference in noradrenaline concentration between aortas from untreated and reserpine pre-treated rabbits (Table 5).

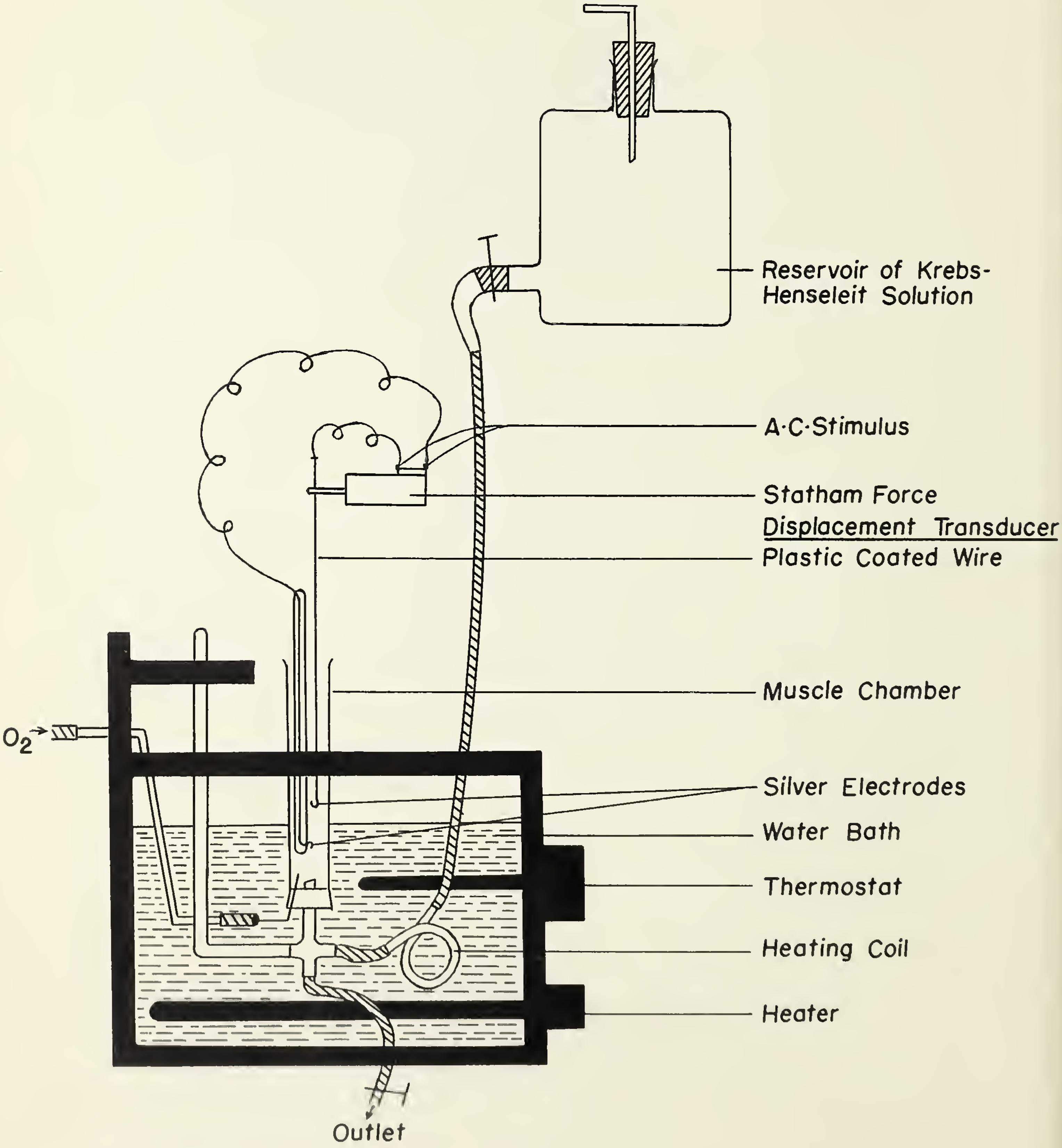
CHAPTER III

EXPERIMENTS ON ISOLATED PREPARATIONS OF RABBIT AORTA AND CAROTID ARTERY

The concentrations of drugs mentioned in this chapter refer to the weight of the drug per millilitre of physiological saline in the organ bath.

Figure II·

Diagram of Apparatus used for Experiments on Isolated Blood Vessels·



METHODS

The effects of sympathomimetic drugs and electrical stimulation upon isolated aorta and carotid artery from untreated and reserpine pre-treated rabbits were investigated. It was hoped to be able to correlate reactivity of vascular strips from untreated and reserpinised rabbits with their respective catechol amine content (pages 23,24).

Apparatus

A water-bath (figure II), thermostatically maintained at 37°C and containing a muscle chamber of about 10 mls. capacity was used.

The muscle chamber was a glass tube 8 cms. long and 2 cms. in diameter with a one-hole rubber stopper at the bottom. An L-shaped glass rod with 3 short pieces of glass tubing fitted at right angles to one another at the foot of the L, was used to fix the organ bath in position. The upper piece of vertical tubing was inserted through the hole in the rubber stopper, the lower vertical piece of tubing connected to an outlet and the horizontal piece of tubing connected, via a heating coil, to an overhead reservoir of Krebs-Henseleit solution (preparation given in Appendix I). The bath could be emptied or washed out by overflow by release of pinchcocks controlling outlet and inlet respectively. The long arm of the L was clamped onto the side of the water bath. Oxygen from a cylinder of compressed oxygen and a reducing valve was passed into the organ bath through tubing attached to a number 18 hypodermic needle pierced through the rubber stopper. The bubble flow was adjusted by means of a pinchcock on the tubing.

The strip of muscle was suspended between 2 silver hooks, the upper of which was soldered to a fine shielded wire threaded through a hole in a small screw designed to screw into the lever of a force displacement transducer. The screw was fixed in position about 10 cms. from the hook, by dipping the wire in a solution of polystyrene in chloroform. The wire was pulled straight by hanging it up and allowing the plastic to dry with the wire under tension. When dry, the wire was passed through a hole in the lever of the transducer and secured by means of the screw. The free upper end of the wire was attached to a source of A.C. current.

The lower hook, which acted as the second electrode, was sealed into the base of a glass tube 7 cms. long and 0.5 cms. in diameter. The upper end of this glass tube had a metal cap through which passed a wire, connected to the hook on the inside of the glass tube and leading to the A.C. source.

The transducer was connected to a rachet device whereby it could be raised and lowered, thus increasing and decreasing the tension on the muscle strip suspended between the two hooks. The glass tube with the lower hook electrode and the transducer with the upper hook electrode was clamped to the same bar fixed into a sliding attachment by which the whole assembly could be clamped onto the edge of the water-bath.

The force displacement transducer was connected to a Grass polygraph. The chart was calibrated by hanging weights from the wire attached to the transducer and noting the deflection of the writing lever. The baseline was adjusted so that the basal tension was 2 gm. for aorta and 1 gm. for carotid artery. Calibration was done at a sensitivity of

1.0 mV/cm., although frequently during the course of an experiment the sensitivity was raised to 0.5 mv/cm., to obtain a larger deflection.

In experiments on the effect of electrical stimulation an automatic timing device was used which stimulated the tissue every 12.5 minutes. A description of this mechanism, which could also cause wash out of the organ bath at regular predetermined time intervals, is given in Appendix IV. An A.C. current was applied via the two hooks inserted one at either end of the muscle strip. The voltage could be regulated by a voltmeter in parallel with the tissue. A potential of 10 or 15 volts for 10 seconds was used to stimulate the aortic and carotid artery strips. Current flow through the tissue could be read off a milliammeter set in series with the tissue. The bathing solution was not removed from the organ bath during electrical stimulation, although this has been recommended by Leonard.(38) Admittedly there is a loss of current through the Krebs-Henseleit due to the high conducting properties of saline solutions, but it was thought that the tissue would remain viable for a longer period of time if it was not removed from the saline. The electrode hooks must be kept scrupulously clean. They tend to become corroded from being immersed in the physiological saline. A black coating develops, which, if not removed by scraping immediately after use, considerably increases the resistance of the system so that very little current flows through the tissue. The wire and hook electrode attached to the transducer had frequently to be replaced, due to the weakness and susceptibility to corrosion of the soldering between copper wire and silver hook.

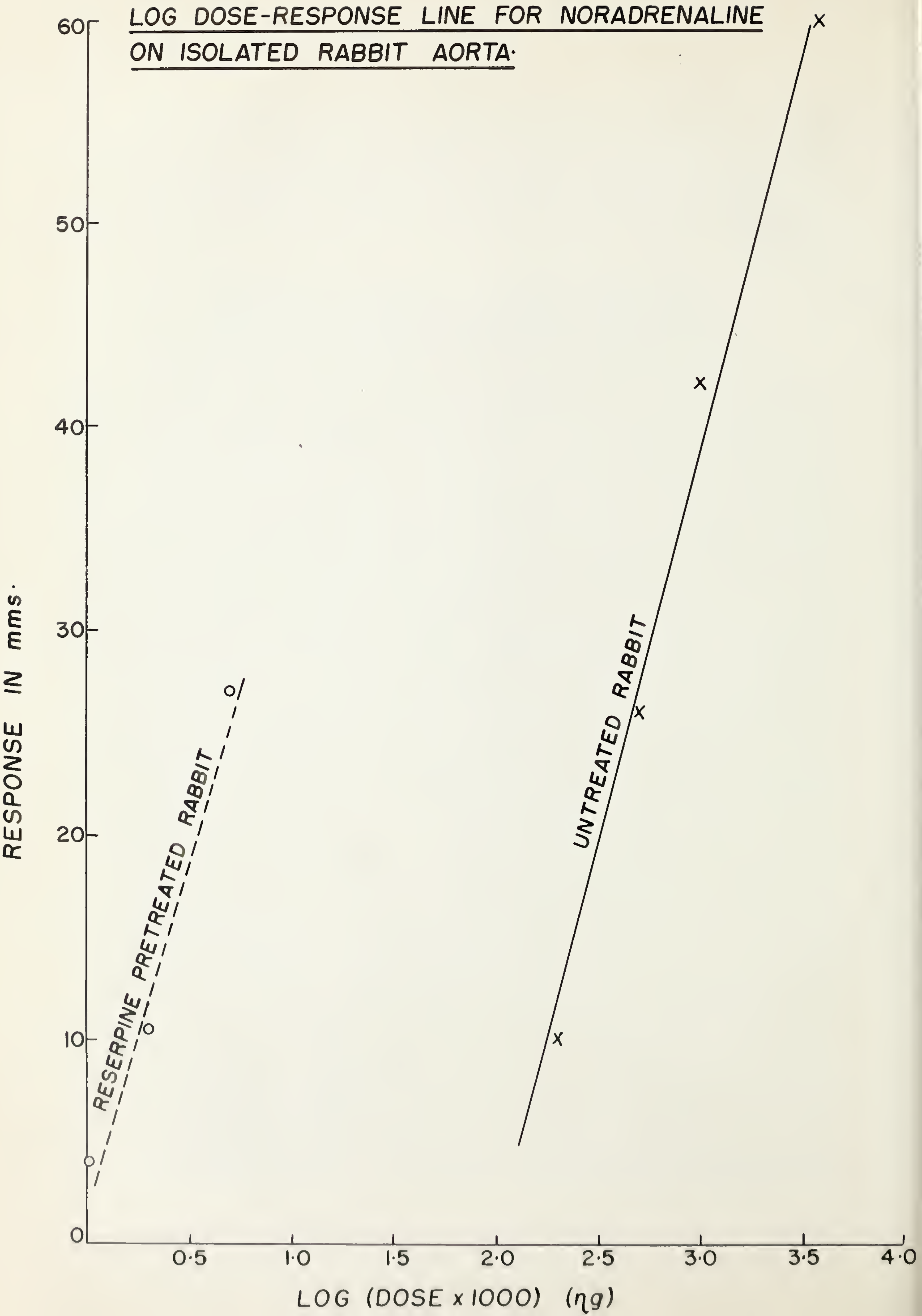
Before a drug was added to the organ bath, the level of physiological saline in the bath was adjusted to a 10 ml. calibration

mark. Drugs were delivered by tuberculin syringes, fitted with number 24 hypodermic needles, in volumes of 0.1 ml. or 0.2 ml.. All drug dilutions added to the bath were prepared in fresh Krebs-Henseleit solution.

Preparation of Aortic and Carotid Artery Strips

Male rabbits were killed by injection of air into the marginal ear vein. The thoracic aorta was removed as quickly as possible after death, taking care to keep it well moistened with Krebs-Henseleit solution and not to stretch it unduly. Fat and connective tissue were trimmed off. The aorta was prepared, after the method of Furchgott and Bhadrakom(9), by cutting it spirally to obtain a strip about 0.4 cms. wide. All aortas were cut in a clockwise direction starting from the diaphragm, although Furchgott(22) found that, in accord with histological examinations which showed an essentially circular orientation of the muscle of rabbit thoracic aorta, there was no significant difference in response of right and left handed spirals of rabbit aorta. A piece about 2.5 cms. long, taken from the upper part of the aorta, was suspended between the two hook electrodes, with the transducer lowered so that the tissue was under no tension. The transducer assembly was then clamped onto the rim of the water-bath, with the tissue completely immersed in Krebs-Henseleit in the organ bath. The tension on the aorta was slowly increased by raising the transducer until a tension of 2 gm., as indicated on the calibrated chart, was reached. The strip was left for 45 minutes before any drugs or electrical stimuli were applied. During this time it relaxed and the tension was readjusted to 2 gm. at intervals by

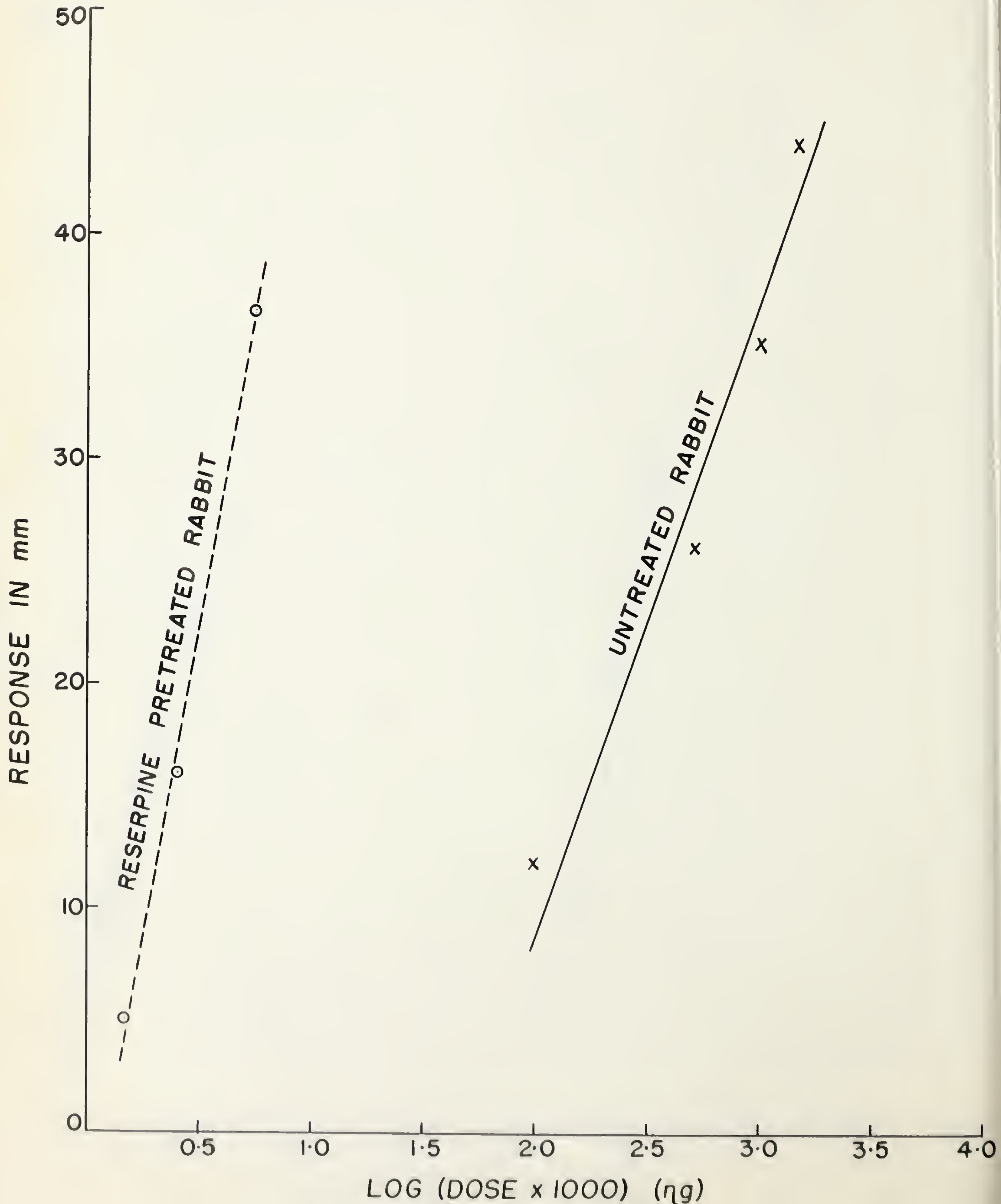
FIGURE IV.



LOG DOSE-RESPONSE LINE FOR NORADRENALINE ON ISOLATED RABBIT AORTA.

FIGURE III·

LOG DOSE-RESPONSE LINE FOR ADRENALINE ON ISOLATED RABBIT AORTA·



raising the transducer. After 45 minutes, depending on the purpose of the experiment, either doses of noradrenaline or adrenaline (see Appendix I for method of preparation of these solutions), or electrical stimuli were applied. Usually, the strip continued to elongate for up to two hours after being placed in the organ bath and it was necessary to recheck the tension to 2 gm. at intervals during this period of time.

The two internal carotid arteries from each rabbit were removed after the aorta. One artery was frozen with liquid nitrogen and its catechol amine content estimated (page 16) and the other, after careful washing with Krebs-Henseleit to free it from blood, stored in a beaker of glucose-free Krebs-Henseleit at 4°C for 24 hours (overnight). After storage, the arteries were removed from the glucose-free Krebs-Henseleit and cut in a clockwise direction, to give strips 0.3 cms. wide and 2.0 to 2.5 cms. long. These strips were mounted in the same fashion as aortic strips, except that a tension of 1 gm. rather than 2 gm. was applied.

RESULTS

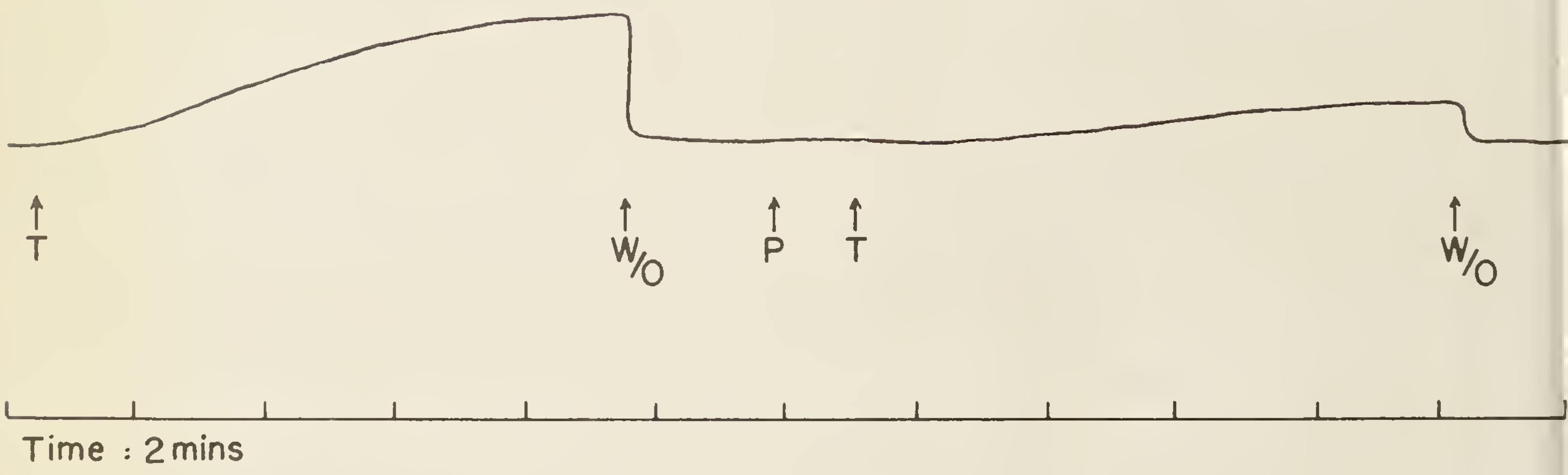
Sensitivity of Aortic Strips to Sympathomimetic Drugs

Adrenaline and Noradrenaline

Linear log dose-response relationships were obtained for the actions of adrenaline and noradrenaline on isolated aortas from untreated and reserpine pre-treated rabbits. As can be seen from figures III and IV, the strips from reserpinised rabbits were about one hundred times more sensitive to the catechol amines, than were the normal strips.

Figure V

EFFECT OF PIPEROXAN ON THE RESPONSE OF ISOLATED RABBIT AORTA TO TOLAZOLINE



At T, Tolazoline 1 µg
At P, Piperoxan 1 µg

Chart stopped after wash-out (W/O)

Before log dose-response relationships could be obtained, a dose of amine giving a sub-maximal response was added at regular intervals to the bath until the sensitivity of the preparation had stabilised. Strips from untreated rabbits showed an increase in sensitivity whereas strips from reserpinised rabbits usually showed a decrease in sensitivity. This decrease in sensitivity was sometimes continuous so that it was impossible to obtain a linear relationship between log dose and response.

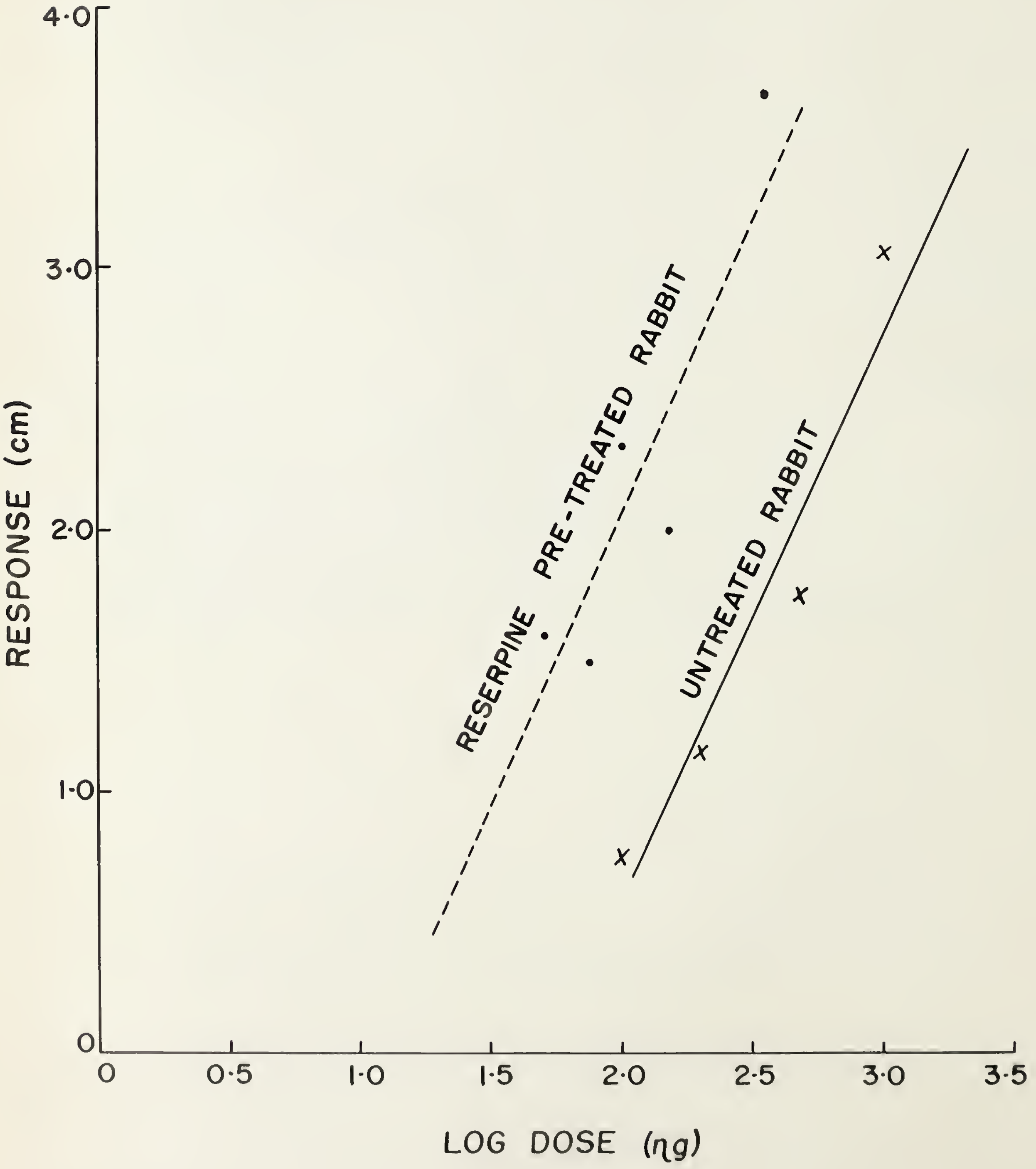
Adrenaline and noradrenaline were approximately equi-active as regards their ability to cause contraction of aortas from normal and reserpinised rabbits (figures III and IV).

Tolazoline

In the course of testing its adrenergic blocking activity, tolazoline was found to cause a slow contraction of the aorta. This contraction could be prevented by the previous addition to the organ bath, of an adrenergic blocking agent such as piperoxan or phentolamine (figure V).

FIGURE VI·

LOG DOSE–RESPONSE LINE FOR TOLAZOLINE ON ISOLATED RABBIT AORTA



Effect of Adrenergic Blocking Agents on the Response of Isolated Aorta to Tolazoline

Expt. No.	State of Aorta	Dose of Tolazoline to Produce Contraction	Dose of Adrenergic Blocking Agent to Block Contraction	
1	from reserpinised rabbit	1 µg	1 µg Piperoxan	Blockade only partially reversible
2		5 ng	0.1 µg "	
3	from untreated rabbit	200 ng	1 µg "	
4		200 ng	1 µg "	
5		1 µg	0.5 µg Phentolamine	irreversible blockade

On plotting log dose-response lines, aortas from reserpinised rabbits were found to be about ten times more sensitive to tolazoline than aortas from untreated rabbits (figure VI). The response of aorta to tolazoline was very slow compared with the response to adrenaline and noradrenaline.

Other adrenergic blocking agents tested, namely phentolamine, piperoxan and dibenzyline, did not show any stimulant action on the isolated aorta.

Sensitivity of Carotid Artery Strips to Sympathomimetic Drugs

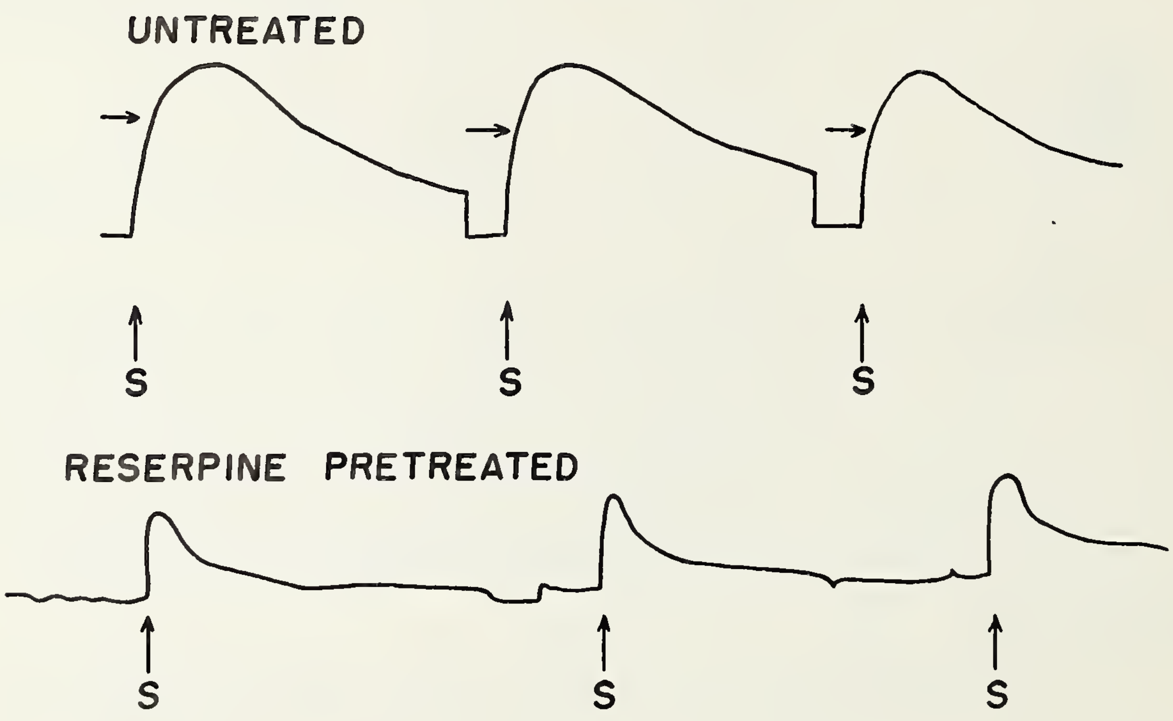
Due probably to being stored overnight, carotid arteries from both treated and untreated rabbits proved to be rather insensitive to adrenaline and noradrenaline.

Response of Aortic Strips to Electrical Stimulation

A dual response to 15 volts A.C. applied for 10 seconds and

FIGURE VII·

THE RESPONSE TO ELECTRICAL STIMULATION OF ISOLATED AORTA FROM UNTREATED AND RESERPINE PRETREATED RABBITS



AT S, 10V A·C· APPLIED FOR 10 SECONDS·

INTERVAL BETWEEN STIMULI, 12·5 MINS·

ARROW INDICATES POINT OF CESSATION OF STIMULUS·

to 10 volts A.C. applied for 10 seconds was obtained from aortic strips removed from 12 and 26 untreated rabbits, respectively. The primary response lasted for the duration of application of the stimulus and a secondary response followed cessation of the electrical stimulus. Aortas from 5 untreated rabbits did not show this secondary response.

The secondary response was abolished by pre-treatment of the animal with the following dose regimens of reserpine:

Dose Regimen of Reserpine	No. of Rabbits Treated
48 hours before use : 2 mg/kg 1/p)) 24 hours before use : 2 mg/kg 1/v)	10
24 hours before use : 2 mg/kg 1/v	10
4 hours before use : 2 mg/kg 1/v	5
2 hours before use : 2 mg/kg 1/v	4

See figure VII.

The primary response, measured as grams tension developed, was compared between series of aortas from treated and untreated rabbits. The current flow was calculated for each strip in milliamperes per centimetre, by dividing the current reading while the tissue was being stimulated with 15 volts, by the length of the particular strip. The width of the strips was kept constant at 0.4 cms. It was found necessary to check the current passing through each strip because even with strips of the same length and width, the current varied. By Ohm's

TABLE 6

PRIMARY RESPONSE TO STIMULATION WITH 15 VOLTS A. C. FOR 10 SECONDS (E. S.) OF ISOLATED AORTA FROM RABBITS UNTREATED AND PRE-TREATED WITH RESERPINE 2 mg/kg 1/v

Expt. No.	Duration of Treatment	Current Flow mA/cm	Mean Current Flow mA/cm ± S.E.	Response to E. S. in gm. tension	Mean Response to E.S. in gm. tension ± S. E.	Signif-cance P
1	Untreated	10.0	13.4 ±1.96	0.4	0.469 ±0.07	
2		10.0		0.65		
3		10.0		0.238		
4		10.0		0.35		
5		19.2		0.5		
6		19.2		0.68		
7	24 hours	10.0	12.8 ±1.7	0.185	0.473 ±0.11	>0.05
8		9.5		0.255		
9		10.0		0.41		
10		11.4		0.35		
11		17.0		0.76		
12		19.2		0.875		
13	4 hours	10.0	10.9 ±1.52	0.19	0.19 ±0.03	<0.05
14		10.0		0.256		
15		7.5		0.13		
16	2 hours	10.0		0.178		
17		9.5		0.08		
18		18.2		0.31		

law, this must be due to changes in the resistance. Such changes can be attributed partly to differences in thickness and internal structure of the tissue and partly to varying degrees of resistance between the tissue and the electrodes due to the ready corrosion of the electrodes (page 27). As would be expected, it was found that, in strips from rabbits subjected to the same treatment, the size of the primary response was approximately proportional to the current flow, expressed as:

total flow in mA / length of strip in cms.

Therefore, for the purpose of comparing the primary response to electrical stimulation of strips from normal and reserpinised rabbits, six strips for each treatment were selected which showed the same range of current flow expressed as mA/cm. (Table 6).

Using the t-test, the primary response of aortas from rabbits pre-treated at 4 hours did not differ significantly from that of aortas from rabbits pre-treated at 2 hours. Therefore, for the purpose of testing for significance of difference between treatments using the F-test, data from the 4 and 2 hour pre-treatments could be pooled. Duncan's multiple range test was used to test which two treatments differed significantly.

The primary response of aortas from untreated rabbits did not differ significantly from the primary response of aortas from rabbits pre-treated at 24 hours. There was a significant difference ($p < 0.05$) between the primary response of normal aortas and of aortas from rabbits pre-treated at 4 and 2 hours.

TABLE 7

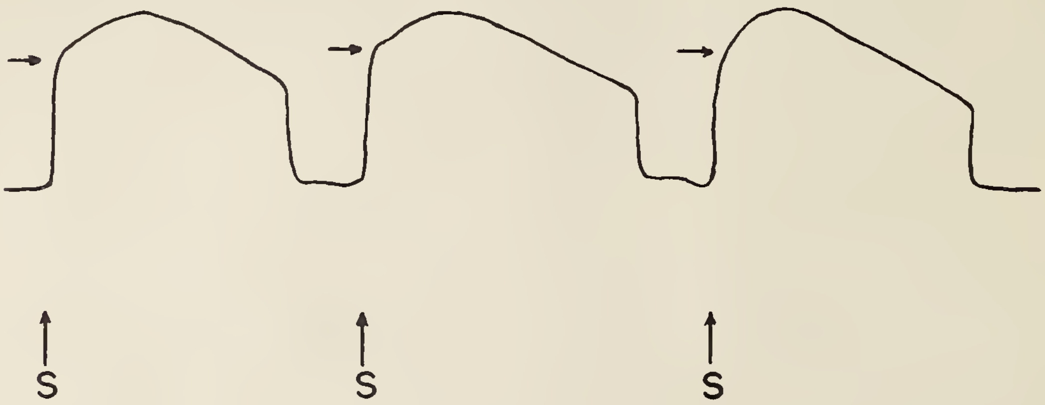
PRIMARY RESPONSE TO STIMULATION WITH 15 VOLTS A. C. FOR 10 SECONDS (E. S.) OF ISOLATED CAROTID ARTERY FROM RABBITS UNTREATED AND PRE-TREATED WITH RESERPINE 2 mg/kg 1/v

Expt. No.	Duration of Treatment	Current Flow mA/cm	Mean Current Flow mA/cm ± S.E.	Response to E. S. in gm. tension	Mean Response to E. S. in gm. tension ± S. E.	Significance P
1	Untreated	16.0		0.125		
2		15.2	16.8	0.136	0.124	
3		16.0		0.225		
4		17.6	±0.72	0.028	±0.03	
5		19.2		0.104		
6	24 hours	17.0		0.23		
7		16.0	17.2	0.11	0.174	
8		16.0		0.10		> 0.05
9		17.0	±0.74	0.10	±0.05	
10		20.0		0.33		
11	4 hours	17.0		0.042		
12		16.0	17.96	0.068	0.063	
13		12.8		0.07		>0.05
14		18.0	±2.19	0.036	±0.02	
15		26.0		0.10		
16	2 hours	17.0		0.05		
17		17.0	16.88	0.083	0.081	
18		18.4		0.095		>0.05
19		16.0	±0.45	0.112	±0.02	
22		16.0		0.068		

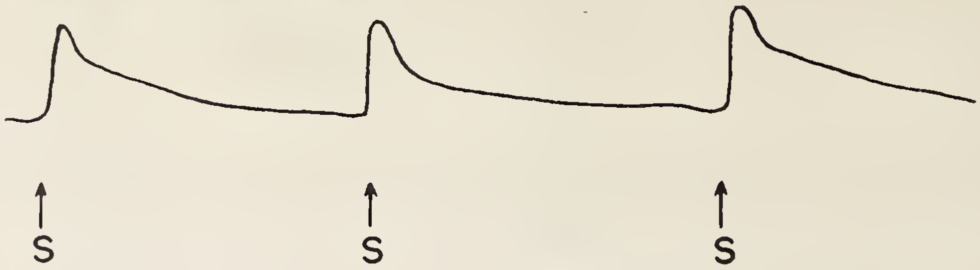
Figure VIII

THE RESPONSE TO ELECTRICAL STIMULATION OF ISOLATED CAROTID ARTERY FROM UNTREATED AND RESERPINE PRETREATED RABBITS

Untreated



Reserpine pretreated



At S, 15V. A.C. applied for 10 seconds.
Interval between stimuli, 12.5 minutes.
Arrow indicates point of cessation of stimulus

Response of Carotid Artery Strips to Electrical Stimulation

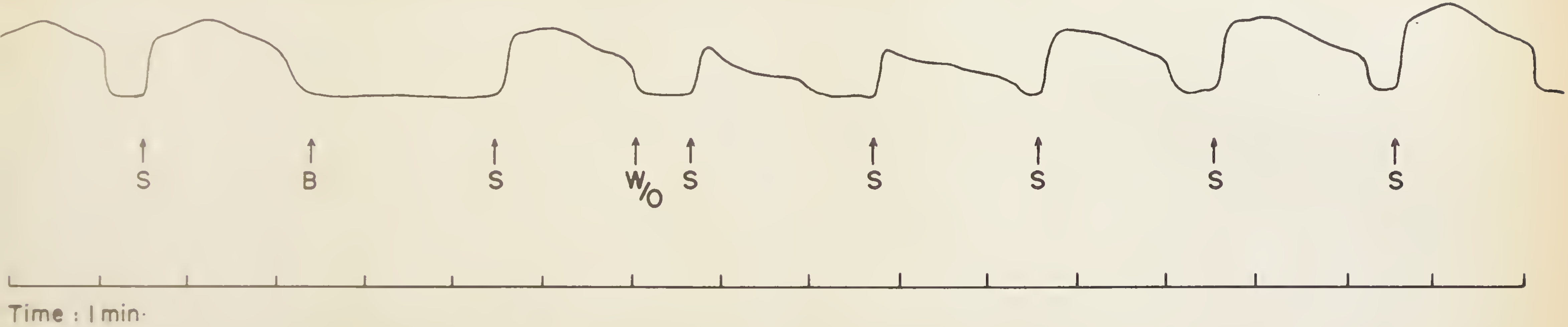
As with aorta, the application of 15 volts A.C. for 10 seconds produced a primary and secondary response of carotid artery strips from untreated rabbits. Pre-treatment with reserpine 2 mg/kg I/V 24 hours, 4 hours and 2 hours before the rabbit was used abolished the secondary response but had no significant effect on the primary response (figure VIII and Table 7). The primary responses of strips from animals treated for different durations were selected and compared using the F-test and Duncan's multiple range test, as described for aortic strips (page 33). These values are considerably lower than those obtained from aortas. There was a higher incidence of strips which responded only slightly or not at all to electrical stimulation among carotid arteries than among aortas. This is probably due to storage of the arteries overnight.

Experiments on the Secondary Response Produced by Electrical Stimulation of Aortic and Carotid Artery Strips from Untreated Rabbits

The fact that reserpine caused depletion of catechol amines stored in aorta and carotid artery (pages 23, 24), suggested that this secondary response, since it did not occur in strips from reserpinised rabbits, may be due to release of catechol amines from the vascular wall. This is substantiated by Furchgott's(28) discovery that addition of the adrenergic blocking agent, dibenamine, to an organ bath containing an aortic strip from a normal rabbit irreversibly abolished the secondary response.

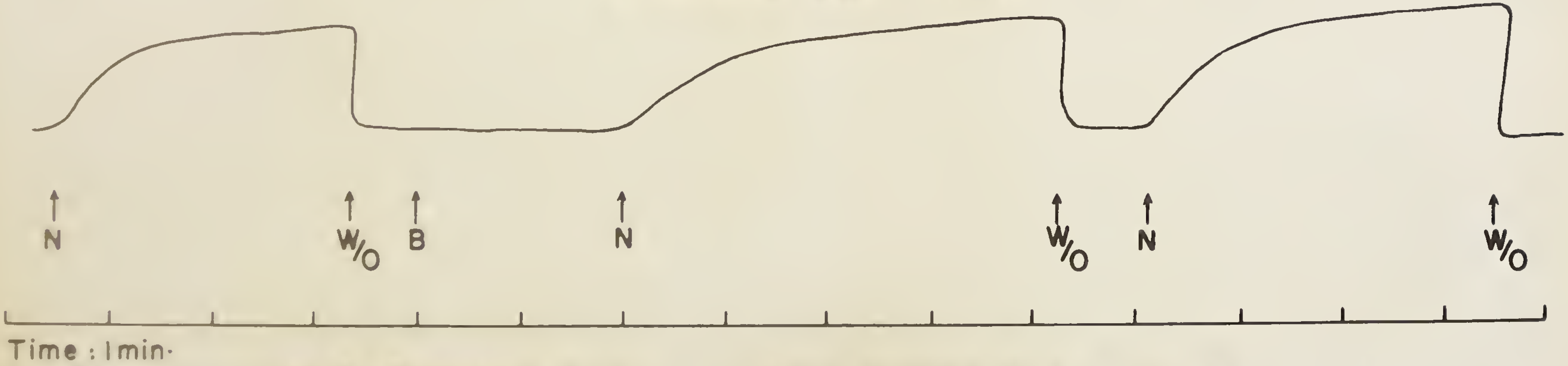
Figure XII

BLOCKADE BY BRETYLIUM, OF THE SECONDARY RESPONSE PRODUCED BY ELECTRICAL STIMULATION OF ISOLATED RABBIT AORTA



At S, 15V.A.C. applied for 10 seconds. Interval between stimuli, 12.5 minutes
At B, Bretylium 1 µg

EFFECT OF BRETYLIUM ON THE RESPONSE TO NORADRENALINE OF ISOLATED RABBIT AORTA

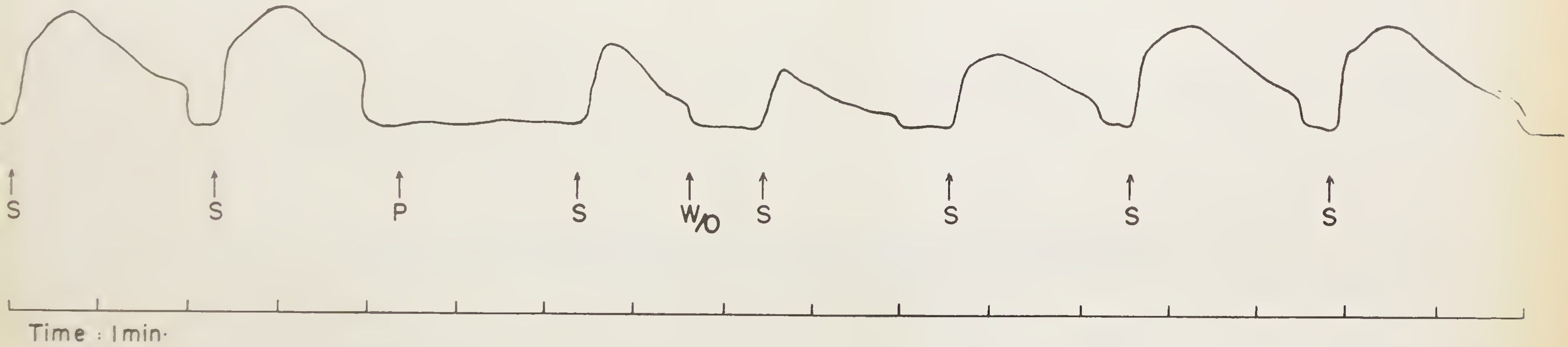


At B, Bretylium 2 µg
At N, Noradrenaline 4 ng

Chart stopped after each wash-out (W/O)

Figure XI

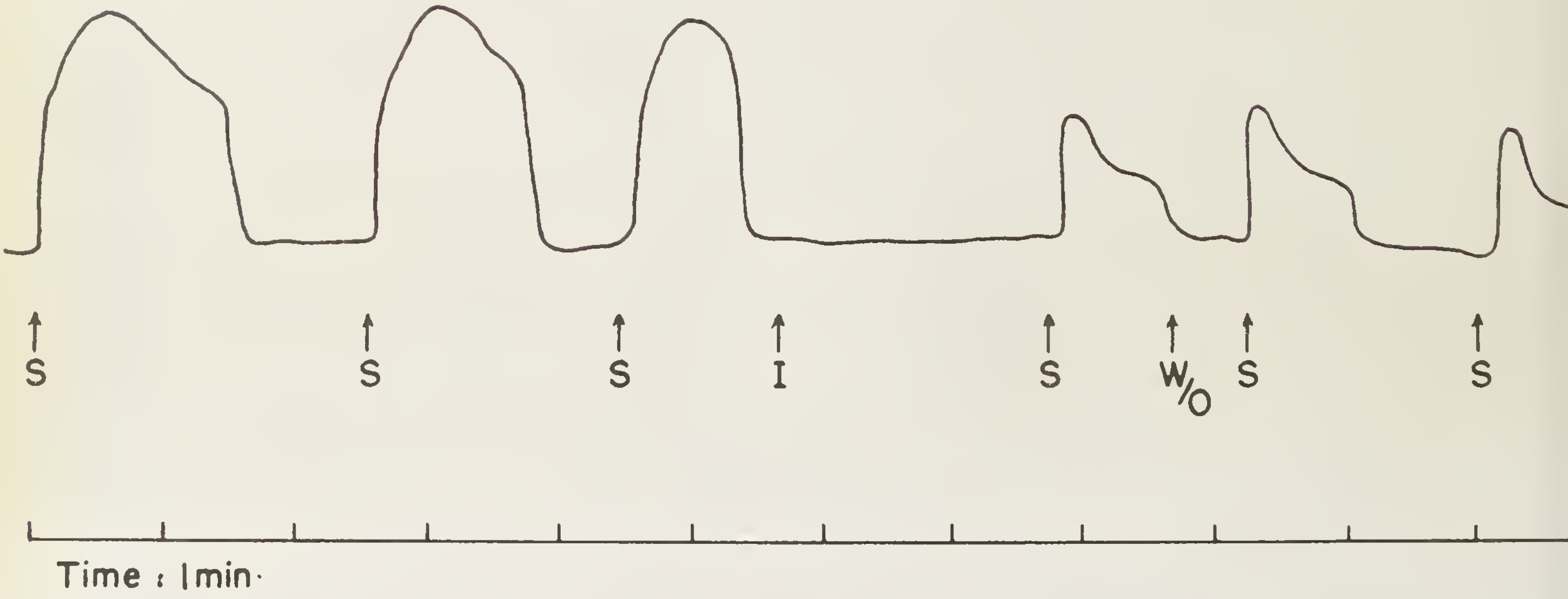
BLOCKADE BY PIPEROXAN, OF THE SECONDARY RESPONSE PRODUCED BY ELECTRICAL STIMULATION OF ISOLATED RABBIT AORTA



At S, 10V. A.C. applied for 10 seconds.
At P, Piperoxan 0.5 μg
Interval between stimuli, 12.5 minutes.

Figure X

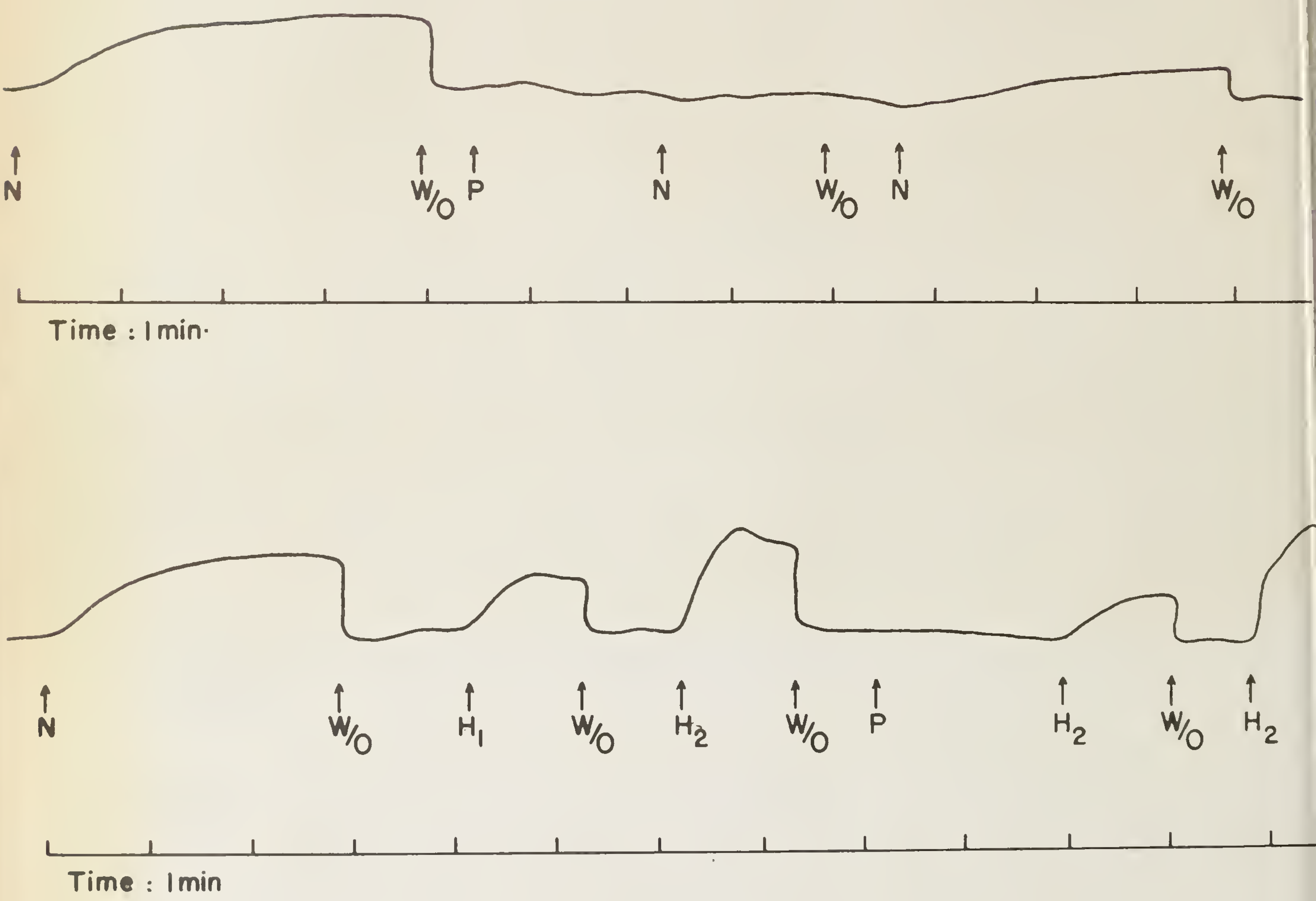
BLOCKADE BY ILIDAR, OF THE SECONDARY RESPONSE PRODUCED BY ELECTRICAL STIMULATION OF ISOLATED RABBIT AORTA



At S, 10V. A.C. applied for 10 seconds.
At I, Ilidar 10µg
Interval between stimuli, 12.5 minutes.

Figure IX

EFFECT OF PIPEROXAN ON THE RESPONSE OF ISOLATED RABBIT AORTA TO NORADRENALINE AND HISTAMINE



At N, Noradrenaline 4 ng
At P, Piperoxan 1 μg

At H_1, Histamine 0.2 μg
At H_2, Histamine 0.3 μg

Chart stopped after wash-out (W/O)

Investigation into the Action of Different Adrenergic Blocking Agents on Aortic Strips

An adrenergic blocking agent which was specific for adrenaline and noradrenaline and which produced a reversible blockade of short duration, was required to test the nature of the secondary response to electrical stimulation. Piperoxan was found to fulfil these requirements. It does have some anti-histamine action but this is probably not very important, as isolated aorta is very much less sensitive to histamine than to noradrenaline (figure IX). Isolated aorta is also relatively insensitive to acetylcholine, 0.4 μg producing a response of equal height as that produced by only 2 ng noradrenaline.

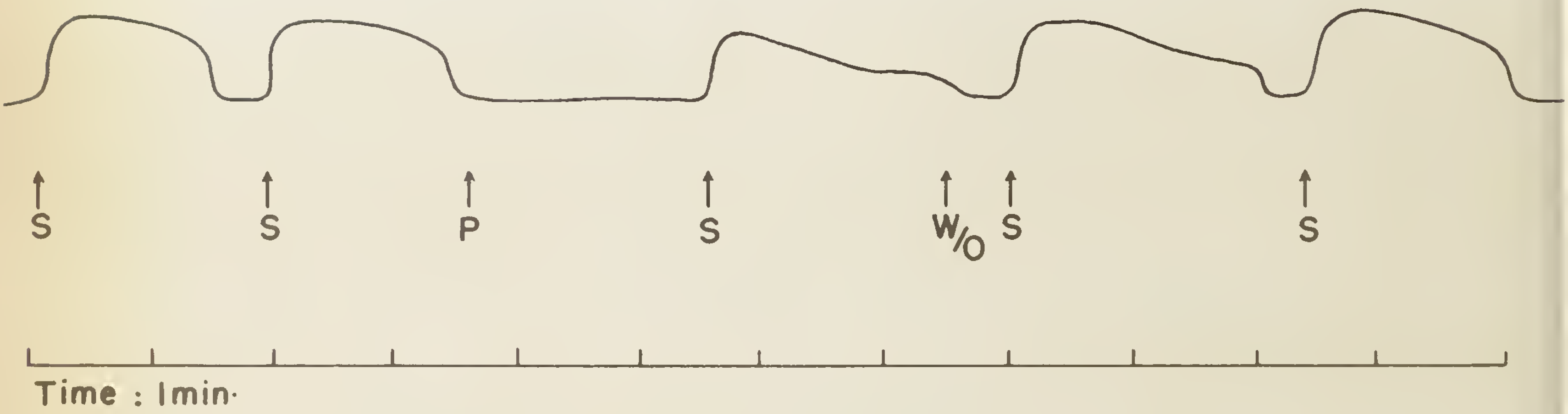
Ilidar, tolazoline and phentolamine were also used. They are specific for catechol amines but have a long duration of action and produce a virtually irreversible blockade. The same can be said for dibenzyline which has in addition the disadvantage of an appreciable anti-histamine activity.

Effect of Adrenergic Blocking Agents on the Secondary Response of Isolated Aorta

The secondary response was irreversibly blocked by ilidar 10 μg in three preparations (figure X). Piperoxan 0.5 μg caused a reversible blockade in three preparations (figure XI). Bretylium 1 to 2 μg reversibly blocked the secondary response in three preparations, although the same dose had no effect upon the response of the aorta to noradrenaline (figure XII). If the dose of blocking agent was increased, some depression of the primary response occurred in addition to complete depression of the secondary response.

Figure XIII

BLOCKADE BY PIPEROXAN, OF THE SECONDARY RESPONSE
PRODUCED BY ELECTRICAL STIMULATION OF
ISOLATED RABBIT CAROTID ARTERY



At S, 15V. A.C. applied for 10 seconds.
At P, Piperoxan 0.5 μg
Interval between stimuli, 12.5 minutes.

Effect of Adrenergic Blocking Agents on the Secondary Response of Isolated Carotid Artery

The secondary response was reversibly blocked by piperoxan 0.5 μg in three preparations (figure XIII) and irreversibly blocked by phentolamine 25 ng in one preparation.

Attempts to Produce a Secondary Response to Electrical Stimulation of Aortic and Carotid Artery Strips from Reserpine Pre-treated Rabbits

If, as the experiments with adrenergic blocking agents suggest, the secondary response in untreated aortas is due to liberation of catechol amines, theoretically it should be possible to produce a secondary response in a reserpinised aorta by repriming the noradrenaline stores by soaking the aorta in catechol amines or their precursors. Burn and Rand(68) have reprimed these stores in reserpinised animals, if restoration of the response to tyramine can be taken to indicate replenishment of the noradrenaline stores. Gillespie and Mackenna(83) abolished the sympathetic effect of stimulating isolated rabbit colon through its extrinsic sympathetic and parasympathetic nerves by pre-treating the rabbits with reserpine. On soaking this preparation in adrenaline, noradrenaline or dopamine, the sympathetic effect could be restored, indicating that some tissues from reserpinised animals are capable of taking up these amines in vitro.

Adrenaline, noradrenaline and dopamine were added to strips of aorta and carotid artery obtained from reserpinised rabbits. The tissue was allowed to recover from the contraction due to the added amine, without washing the amine out of the organ bath. Recovery took

from 30 minutes to 3 hours, depending on the dose used. The responses to electrical stimulation (15 volts A.C. for 10 seconds) were compared before and after addition of the amine. Dopa could not be tested in this way because it proved to be unstable when introduced into the organ bath.

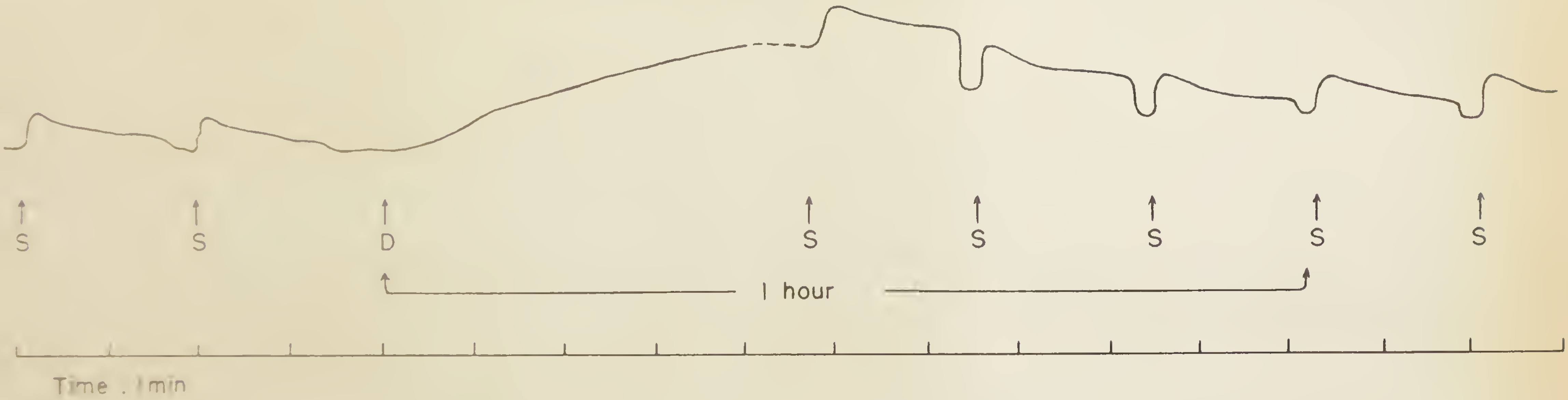
Aortic Strips

Adrenaline, noradrenaline and dopamine were added in the doses indicated:

Expt. No.	Amine Added	Dose
1	Noradrenaline	0.2 ng
2	"	1 ng
3	"	10 ng
4	"	50 ng
5	"	1000 ng
6	Adrenaline	1 ng
7	"	5 ng
8	"	500 ng
9	Dopamine	5 ng
10	"	50 ng
11	"	50 ng
12	"	100 ng
13	"	500 ng
14	"	500 ng

Figure XV

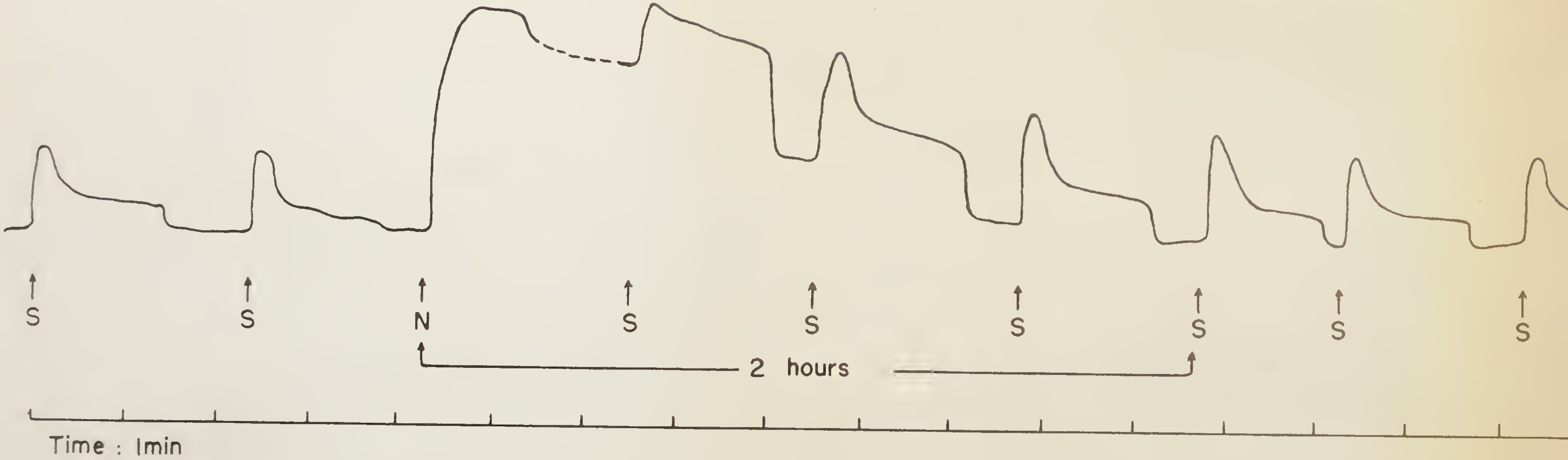
EFFECT OF THE ADDITION OF DOPAMINE ON THE RESPONSE TO ELECTRICAL STIMULATION OF ISOLATED AORTA FROM A RESERPINE PRETREATED RABBIT



At S, 15 V. A.C. applied for 10 seconds.
At D, Dopamine 50 ng
Interval between stimuli, 12.5 minutes.

Figure XIV

EFFECT OF THE ADDITION OF NORADRENALINE ON THE RESPONSE TO ELECTRICAL STIMULATION OF ISOLATED AORTA FROM A RESERPINE PRETREATED RABBIT



At S, 15 V. A.C. applied for 10 seconds.
At N, Noradrenaline 10 ng
Interval between stimuli, 12.5 minutes.

No secondary responses were produced by soaking the aortas in any of the above doses of the three amines, (figures XIV, XV). Dopamine, however, (figure XV) did produce a slight prolongation of the response to electrical stimulation which resembled the slow recovery from electrical stimulation obtained in aortic strips from untreated rabbits. Except when doses of 0.5 μg and more were used which depressed the response, the response to electrical stimulation was unaffected by soaking in adrenaline, noradrenaline or dopamine.

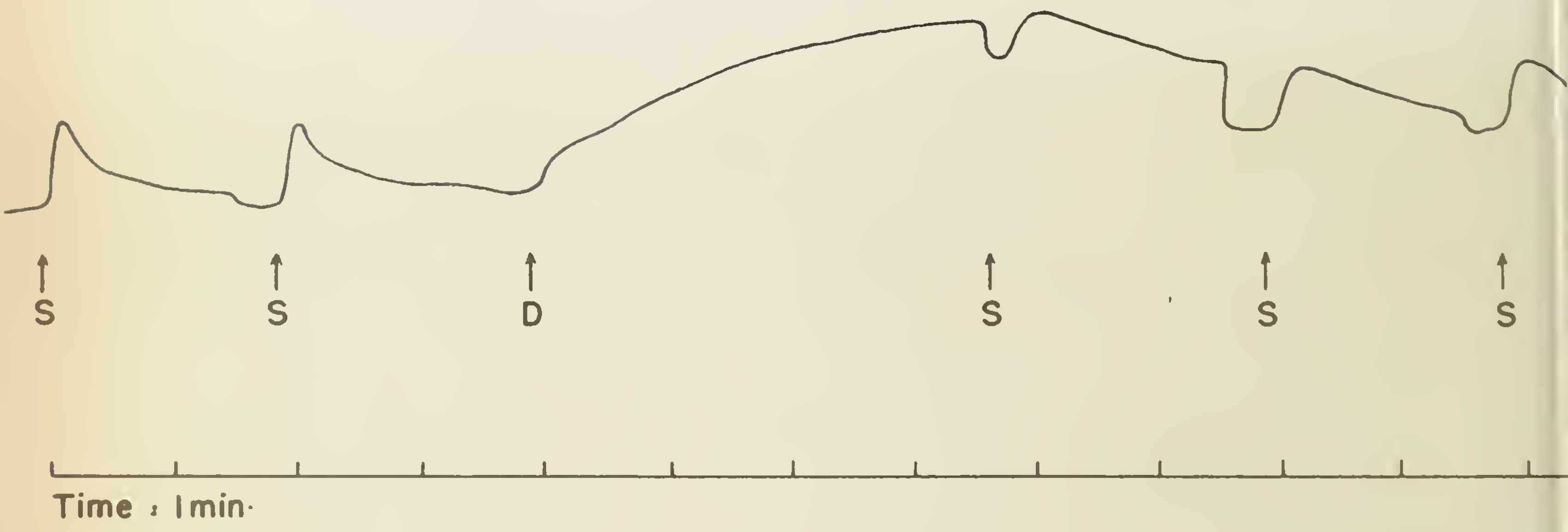
Carotid Artery Strips

Adrenaline, noradrenaline and dopamine were added in the doses indicated:

Expt. No.	Amine Added	Dose
1	Noradrenaline	10 ng
2	"	50 ng
3	"	100 ng
4	"	150 ng
5	"	500 ng
6	Adrenaline	10 ng
7	"	50 ng
8	"	200 ng
9	"	500 ng
10	Dopamine	1 μg
11	"	1 μg
12	"	1 μg
13	"	5 μg
14	"	5 μg
15	"	10 μg

Figure XVI

EFFECT OF THE ADDITION OF DOPAMINE
ON THE RESPONSE TO ELECTRICAL
STIMULATION OF ISOLATED CAROTID ARTERY
FROM A RESERPINE PRETREATED RABBIT



At S, 15 V. A.C. applied for 10 seconds.
At D, Dopamine 10 µg
Interval between stimuli, 12.5 minutes.

No secondary responses were produced by soaking the arteries in any of the above doses of the three amines. As with aorta, dopamine (figure XVI) caused the artery to relax more slowly after the electrical stimulus, although no actual secondary response was produced.

SUMMARY OF RESULTS

1. Pre-treatment of rabbits with reserpine (2 mg/kg l/v 24 hours before use) increased the sensitivity of the isolated aorta to the stimulant actions of adrenaline and noradrenaline by one hundred fold, and to the stimulant action of tolazoline by ten fold.

2. Pre-treatment of rabbits with reserpine (2 mg/kg l/v at 24 hours, 4 hours and 2 hours before use) abolished the secondary response which normally follows cessation of an A.C. stimulus (15 volts for 10 seconds) applied to isolated aorta and carotid artery. Except in the case of aortas from rabbits pre-treated at 4 hours and 2 hours, the primary response of reserpinised strips did not differ significantly from the primary response of normal strips.

3. The secondary response following cessation of an A.C. stimulus (15 volts for 10 seconds) applied to isolated aorta from untreated rabbits was blocked by the addition of piperoxan, ilidar and bretylium to the organ bath. The secondary response of isolated normal carotid arteries was blocked by piperoxan and bretylium.

4. Soaking isolated aortas and carotid arteries obtained from reserpine pre-treated rabbits in adrenaline, noradrenaline or dopamine

did not restore the secondary response to electrical stimulation seen with preparations from untreated rabbits.

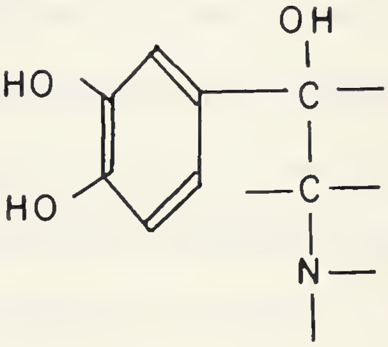
CHAPTER IV

DISCUSSION

DISCUSSION OF THE ASSAY METHOD (CHAPTER II)

Specificity of the Tri-hydroxyindole Fluorimetric Technique

The specificity of the tri-hydroxyindole reaction has been much investigated.(81) It appears to be specific for a catechol nucleus having a 2-carbon chain hydroxylated on the β carbon atom and ending in an amino nitrogen (82) as illustrated. Thus adrenaline, noradrenaline and isopropylnoradrenaline are all assayed using this reaction. Dopamine can be assayed using its tri-hydroxyindole derivative but a modification in the technique normally employed for the estimation of adrenaline and noradrenaline is required.(74) Therefore the presence of some dopamine in tissue extracts will not seriously interfere with the accuracy of determination of adrenaline and noradrenaline. The only amine which might interfere is isopropylnoradrenaline and its presence in any appreciable concentration in biological tissues and fluids has yet to be demonstrated.(90)



In view of the specificity for adrenaline and noradrenaline of this method of assay and also in view of the small amount of material available for extraction it was thought justifiable to use an extraction procedure (page 15) which utilised no purification technique, for the extraction of aorta and carotid artery. Most workers using the tri-hydroxyindole fluorimetric method for the assay of tissue extracts have used some method of purification, but they were working with tissues

which are larger or contain more catechol amine weight for weight(77)(70)(91), than do aorta and carotid artery.

Quenching of the Fluorescence

In applying the formula by which the concentrations of amines are calculated, it is assumed that the fluorescence of adrenolutine and noradrenolutine is additive and that there is no interaction or quenching effect between the two lutines. In aqueous solutions this would appear to be so but with extracts of urine and plasma some degree of quenching may occur. This quenching is reduced by decreasing the volume of sample used for production of the fluorescence.(92) Therefore the volume of the extracting solution should be kept small.

Stability of the Blanks Used in the Fluorimetric Assay

A major problem of the tri-hydroxyindole fluorimetric method is the preparation of stable sample blanks. The reading for the sample blank tends to rise with time, a fact which has been attributed to decomposition of the ascorbic acid in alkali.(92) Sample blanks from extracts which have not been subjected to any purification procedure have high readings and the tendency of such readings to increase further adds appreciably to the error in estimating the fluorescence due to adrenaline and noradrenaline alone. The high readings of such sample blanks can be attributed to light scattering such as can occur from some solutions(93) or from particulate matter not removed by the method of extraction and neutralisation. By reading the reagent blank and the sample blanks of

all extracts assayed, at exactly the same time after addition of the alkali, comparable values should be obtained.

DISCUSSION OF RESULTS OBTAINED WITH THE FLUORIMETRIC AS COMPARED WITH THE BIOLOGICAL METHOD OF ASSAY

Burn and Rand are the only workers to report a value for the catechol amine content of rabbit aorta.(51) Using the blood pressure of a pithed rat, they obtained a mean value of 0.47 μg/gm. for catechol amines estimated as noradrenaline, which agrees quite closely with the mean value of 0.54 μg/gm. obtained by biological assay in this thesis.

No estimates of catechol amines in rabbit aorta assayed fluorimetrically, to compare with the values obtained in Chapter II of this thesis, could be found in the literature. Fluorimetric estimates of catechol amines for other tissues vary widely from author to author and seldom do results obtained using fluorimetric methods of assay agree with results obtained using bio-assays. Thus it was not unprecedented to find that the catechol amine content of normal aorta assayed fluorimetrically (page 23) exceeded the catechol amine content assayed biologically (page 24) by 56%. The higher values obtained with chemical methods of assay are usually attributed to lack of specificity of the chemical, as compared with the biological, method of assay.(94) Results obtained from different laboratories using the tri-hydroxyindole fluorimetric technique to assay the same tissue differ too much for the difference to be explicable solely by variation between animals of the concentration of amine in that tissue. Weil-Malherbe(95) and Crout(92)

suggest that individual variation between laboratories in techniques (for instance in the times at which the samples are read - see page 19) and instrumentation may account for these differences. Although the fluorimetric methods may be suspect as regards determination of the absolute concentrations of catechol amines in plasma and tissue, provided all estimations are carried out in the same laboratory and using exactly the same technique, the methods are sufficiently reproducible and sensitive to detect differences in amine concentration between tissues.

GENERAL DISCUSSION

According to Burn and Rand's hypothesis, the catechol amine released from tissues by reserpine is noradrenaline, which, before release, is stored by the tissue in certain noradrenaline stores. Schmiterlöw(40) and Burn and Rand(51), using qualitative tests, found that the catechol amine content of horse and rabbit aorta respectively, is composed largely of noradrenaline. It was not found possible to verify this quantitatively using the tri-hydroxyindole method to assay the concentrations of adrenaline and noradrenaline separately in extracts of rabbit aorta, due to the very low concentrations of the amines in such extracts. In view of the observation of Paasonen and Krayer(53), that reserpine causes a selective depletion of noradrenaline from rat hearts, it would appear that the amine dispersed from vascular tissue by reserpine is most likely to be noradrenaline.

In experiments described in this thesis, pre-treatment of rabbits with reserpine (2 mg/kg l/v) 24 hours before use increased the

sensitivity of the isolated aorta to the actions of adrenaline and noradrenaline at the same time as causing a decrease in the catechol amine content. The sensitivity to sympathomimetic amines of aortic strips from reserpine pre-treated rabbits, instead of showing the slow rise to a steady maximum over a period of time typical of normal strips, showed a decline which was often continuous. Burn and Rand(51) attributed the increased sensitivity to adrenaline and noradrenaline following reserpinisation to depletion of the tissue stores of noradrenaline, with the consequent availability of more free receptors with which the added amine can react. The decline in sensitivity over a period of time of strips from reserpinised rabbits could be due to such strips taking up the added amine in an effort to fill their noradrenaline tissue stores which have been depleted by reserpine. The catechol amine content of aorta was also reduced by pre-treatment with reserpine for 4 and 2 hours respectively. Unfortunately, insufficient investigations of aortas from rabbits pre-treated for these periods of time were carried out to determine if their sensitivities to the actions of adrenaline and noradrenaline were increased. This would be expected if Burn and Rand's hypothesis is valid. Paasonen and Krayer(53) found a significant reduction in the noradrenaline content of rat hearts to occur after only 2 hours' treatment with reserpine. They did not, however, investigate the sensitivities of such rat hearts to adrenaline and noradrenaline. Using dog heart lung preparations, they found that reserpine caused a pronounced chronotropic action before there was any change in the catechol amine content of the dog hearts. Innes(62) found enhanced responses to adrenaline and noradrenaline before any depletion of

catechol amines in the nictitating membrance had occurred. Thus from the literature a depletion of tissue noradrenaline does not seem to be a prerequisite for an increased sensitivity to noradrenaline. Whether an increased sensitivity to noradrenaline invariably follows a lowering of tissue noradrenaline would appear to be undecided.

The secondary response which normally follows cessation of application of an A.C. stimulus to aortic strips did not occur in aortas from rabbits pre-treated with reserpine for 24, 4 or 2 hours. The catechol amine content of these aortas was significantly less than the catechol amine content of normal aortas. In addition, the response of aortas from reserpinised rabbits to electrical stimulation closely resembled the response to electrical stimulation of normal aortas after an adrenergic blocking agent. This evidence strengthens Furchgott's suggestion(28) that the secondary response is due to liberation of an adrenaline-like substance, which is most probably noradrenaline (page 44). A reversible blockade of the secondary response of normal aortas could be achieved with doses of piperoxan which did not affect the primary response. The same doses completely inhibited the action of noradrenaline on the aorta but only partly inhibited the action of histamine on the aorta. These facts suggested that the effect of piperoxan on the secondary response was specific to block the action of some noradrenaline-like substance liberated by the electrical stimulus. Reversible blockade of the secondary response produced by bretylium was not due to successful competition by bretylium with released noradrenaline for tissue receptors, because the same dose of bretylium did not reduce the action of added

noradrenaline on the isolated aorta. Bretylium depresses the peripheral sympathetic nervous system by inhibition of conduction along the nerves, the finer nerve endings being more susceptible to this action than the nerve trunks.(96) Thus the blocking action of bretylium on the secondary response suggested that electrical stimulation of aorta releases noradrenaline from the tissue stores by stimulation of nerve fibres in the aorta wall. The onset of the blockade was more rapid than that which has been reported for bretylium on isolated nerve preparations.(96) This difference in speed of action could be due to the different preparations. It is unlikely, in view of the recovery of the secondary response after bretylium, that the blockade is of a non-specific nature. An alternative explanation which, however, does not take into account the postulated action of bretylium, is that the electrical stimulus acts directly on the noradrenaline tissue stores to release the amine. Since there is no evidence that noradrenaline may be located outside nervous tissue(97), these noradrenaline stores must lie either within the sympathetic nerve endings or in chromaffin cells within the tissue.(98) Schmiterlöw found that the amount of noradrenaline in aorta is greatest in the outer part of the aorta wall where the nerves enter. This fact, together with the observation that denervation of organs causes a decrease in their noradrenaline content(99)(100), suggests that tissue noradrenaline is most likely stored within the sympathetic endings which contain far larger amounts of noradrenaline per unit weight than the pre-terminal axons(97), or in chromaffin cells closely associated with the nerve endings. The manner in which the noradrenaline is held in these

stores is not known. If the stores are situated at the nerve endings, it is probable that noradrenaline is contained within granules similar to the chromaffin granules of the adrenal medulla(101), which have been demonstrated in sympathetic nerve fibres(102). Dispersal of noradrenaline from the granules by an electrical stimulus could be effected by increasing the permeability of the granules. Although these stores have not been demonstrated histologically, something like them must exist to account for the ability of tissues to store noradrenaline in a pharmacologically inactive form. Burn and Rand maintain that the noradrenaline in these stores, although inactive, is not immobile and can pass out of the stores and react with tissue receptors. This is possibly an important factor in the maintenance of basal vascular tone.(61)

Continued electrical stimulation of strips of aorta from normal and reserpine pre-treated rabbits for several hours resulted in no significant change in the aortic concentration of catechol amines. This was as would be expected for the strips from reserpinised animals in which there was no release of catechol amines upon electrical stimulation, as judged by the absence of a secondary response. Stimulation of normal aortas may release such infinitesmal amounts of catechol amine that even after stimulating for many hours, no reduction in the tissue amine concentration can be detected by the methods of assay available.

When given 24 hours before the rabbit was used, the action of reserpine was specifically upon the noradrenaline stores, in that there was a significant reduction in the catechol amine content of the aorta

and the secondary response was abolished without the primary response (due to the direct effect of the electrical stimulus upon the aortic smooth muscle) being affected. Pre-treatment at 4 and 2 hours before use also significantly lowered the catechol amine concentration of the aorta, but in addition to abolishing the secondary response, caused a significant reduction in the primary response. This latter effect was presumably due to a direct interference by reserpine with the contractile mechanism activated by the electrical stimulus. A 50 percent reduction of the response to electrical stimulation of aortic strips has been produced by oxygen-free conditions.(28) It is therefore possible that reserpine can cause reduction in the response to electrical stimulation due to the interference it has been postulated to cause in the oxidative metabolism of aortic smooth muscle.(48)(49) The reason for the absence of an effect on the primary response after pre-treatment at 24 hours could be that the aorta has had time to recover from the depressant action of reserpine before being removed from the animal.

Reserpine treatment of animals appears to render their tissues even less ready to take up noradrenaline from intravenous infusions than are tissues from untreated animals.(103)(67)(104) Pennefather and Rand(69) suggest that this may indicate an interference by reserpine with the binding mechanism for noradrenaline. Nevertheless, a response to tyramine in reserpinised animals can be produced by an intravenous infusion of noradrenaline(67)(105), suggesting that some noradrenaline must be taken up by the tissues. The amount necessary to restore the response

to tyramine may be too small to be detected by the available methods of assay. This could account for Muscholl's(67) failure to find any increase of noradrenaline in the heart muscle of a rat previously treated with reserpine, after the intravenous infusion of amounts of noradrenaline which were sufficient to restore the pressor action of tyramine. The amount of noradrenaline required to be taken up by aortas from reserpine pre-treated rabbits in order to produce a secondary response to electrical stimulation may be very much larger than that required to be taken up to restore the response to tyramine. Although the amount of noradrenaline responsible for one secondary response must be very small (page 48), the marked difference between the catechol amine concentrations of aortas from normal and reserpinised rabbits, together with the fact that none of the aortas from the reserpinised rabbits showed a secondary response, suggest that in order for a secondary response to occur, the catechol amine concentration must be above a certain minimum level. This level will presumably vary from animal to animal. The inability to produce secondary responses to electrical stimulation of aortas from reserpinised rabbits by soaking the preparations in adrenaline, noradrenaline and dopamine may be due to insufficient uptake of the amines. This could be caused by reserpine interfering with the binding mechanism of the amines. In addition, there may be only poor penetration of in vitro blood vessels by these amines. Of interest in this regard is Burn and Rand's observation(105) that an infusion of noradrenaline into a reserpine pre-treated animal restored the action of tyramine to a greater extent than it restored the effect of sympathetic stimulation.

The stimulant action of tolazoline upon isolated aorta, since it was increased by pre-treatment of the rabbits with reserpine and was reversibly blocked by an adrenergic blocking agent, would appear to be mediated via the sympathetic receptors. It is noteworthy that Ahlquist(106) and Gowdey(107) reported that tolazoline constricts the coronary arteries of the rabbit, an action they attributed to the histaminic activity of tolazoline. Innes(108) demonstrated that another adrenergic blocking agent, ergotamine, exerts a stimulant action on the smooth muscle of aorta via the adrenergic receptors.

There are no reports in the literature on the effects of reserpine pre-treatment on the catechol amine content of rabbit carotid artery. In experiments recorded in this thesis the difference between the catechol amine concentration of carotid arteries from untreated and from reserpinised rabbits was more marked than that found between aortas from the same rabbits. This may be due to a higher catechol amine content of the artery as compared with the aorta, or to a decreased loss of the amines from the artery which, unlike the aorta, was frozen immediately upon removal from the animal. As with aorta, along with a reduction in the catechol amine content of the carotid artery, reserpine pre-treatment of the rabbit 24, 4 or 2 hours before use, abolished the secondary response which normally followed cessation of an A.C. stimulus applied to artery strips from untreated rabbits. Unlike the aorta, the primary response to electrical stimulation of carotid arteries from rabbits pre-treated 4 and 2 hours before use did not differ significantly from the primary response of normal carotid arteries. It is unlikely

that storing the tissue overnight could account for this difference between aorta and carotid artery. Possibly the depressant effect of reserpine upon the response to electrical stimulation was more apparent in the aorta than in the carotid artery because the former has a lower proportion of smooth muscle than has the latter.(34) Leonard(38) found that a secondary response was produced by electrical stimulation of carotid artery but he did not investigate the nature of this response. It was found in the work reported here that this secondary response could be reversibly blocked by piperoxan and bretylium and that no secondary response could be produced in artery strips from reserpinised animals by soaking such strips in adrenaline, noradrenaline or dopamine. It can therefore be inferred that the secondary response to electrical stimulation of normal carotid artery strips is of the same nature as the secondary response to electrical stimulation of normal aortic strips. The mechanisms involved in the release of noradrenaline from the noradrenaline stores by reserpine and electrical stimulation appear to be the same in both blood vessels. The sensitivities of carotid artery to adrenaline and noradrenaline were not investigated because the arteries were all stored overnight. This treatment seemed to reduce their sensitivities to sympathomimetic amines although the response to A.C. stimulation was unaffected. This same effect was noticed by Meyer(8), using cow carotid artery rings.

CONCLUSIONS

1. The increased sensitivity to the actions of adrenaline and noradrenaline on aortic strips from reserpinised rabbits is associated with a depletion by reserpine of noradrenaline stores in the aorta wall.

2. The stimulant action of tolazoline on isolated aorta is mediated by activation of adrenergic receptors.

3. The secondary response which follows cessation of an A.C. stimulus to isolated aorta and carotid artery from normal rabbits is due to release of noradrenaline from the noradrenaline stores in the respective blood vessels. It is postulated that the electrical stimulus effects this release by stimulation of nerve fibres in the blood vessel wall.

It was not possible to reprime the noradrenaline stores in isolated aorta and carotid artery from reserpinised rabbits, by prolonged soaking of the blood vessels in noradrenaline, adrenaline and dopamine.

4. Using the tri-hydroxyindole fluorimetric method, the concentrations of catechol amines, expressed as noradrenaline free base, in rabbit aorta and carotid artery were estimated to be 0.86 μg/gm. and 1.88 μg/gm., respectively.

5. Pre-treatment of rabbits with reserpine significantly reduced the concentration of catechol amines in both aorta and carotid artery.

6. The tri-hydroxyindole fluorimetric method for the assay of catechol amines in tissue extracts cannot detect accurately amounts of amines of less than 0.02 μg per sample. Adrenaline and noradrenaline cannot be assayed differentially using this method, if the amount of either amine present in the sample is less than 0.1 μg.

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APPENDICES

APPENDIX I

PREPARATION OF REAGENTS AND DRUGS

All reagents used were of Analar quality and were prepared in de-ionised water, unless otherwise stated.

Adrenaline 10 mg/ml. - 200 mg. Adrenaline bitartrate (Suprarenin bitartrate - Winthrop) in 0.05% Sodium metabisulphite.

Bromothymol blue 0.1% - 1 gm. triturated in a mortar with 16 mls. 0.1N Sodium hydroxide and adjusted to 1 litre with water.

Hydrochloric acid 0.1N - 5 mls. 2N HCl in 100 mls. water

Noradrenaline 10 mg/ml. - 200 mg. Noradrenaline bitartrate (Levarterenol-Winthrop) in 0.05% Sodium metabisulphite.

Perchloric acid 0.3N - 5 mls. 60% $HClO_4$ in 100 mls. water.

Phosphate buffer 0.1M pH 6.5 - 80 mls. 0.067M KH_2PO_4 (0.9078gm/100mls.) mixed with 20 mls. 0.067M $Na_2HPO_4.12H_2O$ (2.39gm/100mls.)

Potassium carbonate 0.8N - 14.48 gm. $K_2CO_3.1\text{-}1/2H_2O$ in 100 mls. water.

Potassium ferricyanide 0.25% - 0.25 gm $K_3Fe(C_N)_6$ in 100 mls. water and stored in a dark bottle at 4°C.

Sodium hydroxide 5N - 245 gm. NaOH pellets in 100 mls. water.

Trichloroacetic acid 10% - 10 gm. CCL_3COOH in 100 mls. water.

Trichloroacetic acid 5% - 10 mls. trichloroacetic acid 5% plus 10 mls. normal saline.

Preparation of Krebs-Henseleit Solution

Sodium chloride 25%	138.4 mls.
Potassium chloride 15%	11.6 mls.
Calcium chloride 10%	14.5 mls.
Potassium dihydrogen phosphate 5%	16.0 mls.
Magnesium sulphate 10% (heptahydrate)	14.5 mls.

Preparation of Krebs-Henseleit Solution - Cont'd.

Sodium bicarbonate 5%		119.0 mls.
Calcium disodium versenate 5%		1.0 ml.
Glucose		10.0 gm.
De-ionised water	to	5.0 litres.

The above stock solutions, with the exception of the sodium bicarbonate which must be renewed every two weeks, will keep for many months without deterioration.

Calcium disodium versenate was included to protect added adrenaline and noradrenaline from inactivation by any metallic ions present in the bathing medium.

APPENDIX II

CALCULATIONS OF RESULTS IN CHAPTER II - ESTIMATION OF CATECHOL AMINES IN VASCULAR EXTRACTS

ESTIMATION OF CATECHOL AMINES USING THE TRI-HYDROXYINDOLE FLUORIMETRIC METHOD

The Aminco-Bowman spectrophotofluorimeter with the following settings was used for all readings quoted:

Slit No. 5, Sensitivity maximum, fluorescence wavelength 510 $m\mu$.

Example of Calculation of Fluorescence Reading

e.g. A 0.1 μg/ml. activated at 400 $m\mu$

	% fluorescence scale reading	meter multiplier reading	final reading
A	52.5	0.3	52.5 x 0.3 = 15.75
Blank A	11.0	0.03	11.0 x 0.03 = 0.33

Therefore % fluorescence due to A = 15.75 - 0.33

= 15.42 (indicated as A_{400})

Calculation of Average Standards

		A		NA	
Activation Wavelengths		400$m\mu$	445$m\mu$	400$m\mu$	445$m\mu$
Date of prep. of stock solutions	Date of readings				
14.3.61	15.3.61	15.02	10.99	15.25	3.33
"	16.3.61	14.42	10.52	16.07	3.92
"	17.3.61	15.48	10.5	15.98	3.79
27.3.61	27.3.61	15.42	10.85	14.88	3.38
"	18.4.61	15.4	9.4	15.8	2.8
Freshly prepared solutions:					
I	20.3.61	15.5	10.7	15.61	3.44
II	"				
Average		15.21	10.49	15.6	3.44
Standard Deviation		0.42	0.57	0.47	0.4

Calculation of Adrenaline and Noradrenaline in Aorta (or Carotid Artery)

(1) The concentrations of adrenaline and noradrenaline in the final fluorescent solution were calculated by the following formula:

$$S_{400} = yN_{400} + xA_{400}$$

$$S_{445} = yN_{445} + xA_{445}$$

where S_{400} = fluorimetric reading of sample at 400 mμ per μg/ml.

S_{445} = " " " " " 445 mμ per μg/ml.

N_{400} = averaged reading for fluorescence of noradrenaline at 400 mμ activation per μg/ml.

N_{445} = averaged reading for fluorescence of noradrenaline at 445 mμ activation per μg/ml.

A_{400} = averaged reading for fluorescence of adrenaline at 400 mμ activation per μg/ml.

A_{445} = averaged reading for fluorescence of adrenaline at 445 mμ activation per μg/ml.

(2) The total amines were expressed as noradrenaline by the use of the following formula:

$$y' = \frac{S_{400}}{N_{400}}$$

where y' = adrenaline plus noradrenaline in the sample fluorescent solution expressed as noradrenaline in μg/ml.

For example:

For an extract S, obtained by extraction of the aorta from an untreated rabbit.

Weight of aorta = 84.5 mg.

Volume of extract solution = 2.7 mls. (2 mls. perchloric acid plus 0.7 ml. potassium carbonate)

$S_{400} = 0.63$ $S_{445} = 0.3$

(I) Differential assay:

$A_{400} = 15.21$ $N_{400} = 15.6$

$A_{445} = 10.49$ $N_{445} = 3.44$

$S_{400} = yN_{400} + xA_{400}$

$S_{445} = yN_{445} + xA_{445}$

by substitution in these simultaneous equations,

$$0.63 = \frac{15.6}{0.1}y + \frac{15.21}{0.1}x$$

$$0.3 = \frac{3.44}{0.1}y + \frac{10.49}{0.1}x$$

(standards are 0.1µg/ml. therefore to express x and y as µg/ml. it is necessary to divide by 0.1)

By solution of these simultaneous equations,

x = 0.002213 (µg A per ml. fluorescent solution)

y = 0.00188 (µg NA per ml. fluorescent solution)

1 ml. S was adjusted to 10 mls. in the production of fluorescence and volume of extract solution S = 2.7 mls.

Therefore A extracted = 0.002213 x 10 x 2.7

but this is contained in 84.5 mg. aorta.

Therefore 1 gm. aorta contains $\frac{0.002213 \times 10 \times 2.7 \times 1000}{84.5}$ µg A

= 0.71 µg A

= 0.39 µg A base

Similarly,

1 gm. aorta contains 0.32 µg NA base

In a few cases a negative result or negative results for A and/or NA were obtained upon solution of the simultaneous equations.

This occurred more frequently with extracts from reserpinised rabbits, suggesting that such negative results signify concentrations too low to be measured. Negative results occurred only in the differential method of estimation; using the method for total catechol amines positive values could always be obtained, even for those extracts giving negative results by the differential method.

(2) Calculation of total catechol amines expressed as noradrenaline:

Concentration of C.As, expressed as NA in the fluorescent solution =

$$\frac{0.1 \times 0.63}{15.6} \qquad (S_{400} = 0.63)$$

Hence 1 gm. aorta contains C.As equivalent to:

$$\frac{0.1 \times 0.63 \times 10 \times 2.7 \times 1000}{15.6 \times 84.5}$$

$$= 1.29\ \mu\text{g NA}$$

$$= \underline{0.68\ \mu\text{g NA base}}$$

Calculation of Recovery of Adrenaline or Noradrenaline Added to Aorta Prior to Extraction

e.g. S_O - extract from half the aorta with no adrenaline or noradrenaline added.

S_A - extract from the other half of the aorta with 1 μg adrenaline added before extraction.

From S_O:

1 gm. aorta contains 0.59 μg A plus 0.49 μg NA

From S_A:

$x = 0.0246\ \mu$g A per ml. fluorescent solution

1 ml. S_A was adjusted to 10 mls. and volume of extract solution = 2.8 ml.

Therefore A extracted = $0.0246 \times 10 \times 2.8$ µg

$= 0.69$ µg

Weight of aorta extracted to give $S_A = 92.5$ mg.

1 gm. aorta contains 0.59 µg A (from S_O)

Therefore 92.5 mg. aorta contains $\frac{0.59 \times 92.5}{1000}$ µg A

$= 0.054$ µg A

Therefore recovery of A = $0.69 - 0.054$ µg

$= 0.64$ µg

Since 1 µg A was added,

Recovery of A = $\frac{0.64 \times 100}{1.0}$

$= \underline{64\%}$

Recoveries of Adrenaline and Noradrenaline taken through the Extraction and Assay Procedure (table 8)

and

Differential Estimation of Adrenaline and Noradrenaline (table 9)

The following method for expressing the amount of A and NA added, in terms of NA was used:

$N_{400} = 15.6$

$A_{400} = 15.21$

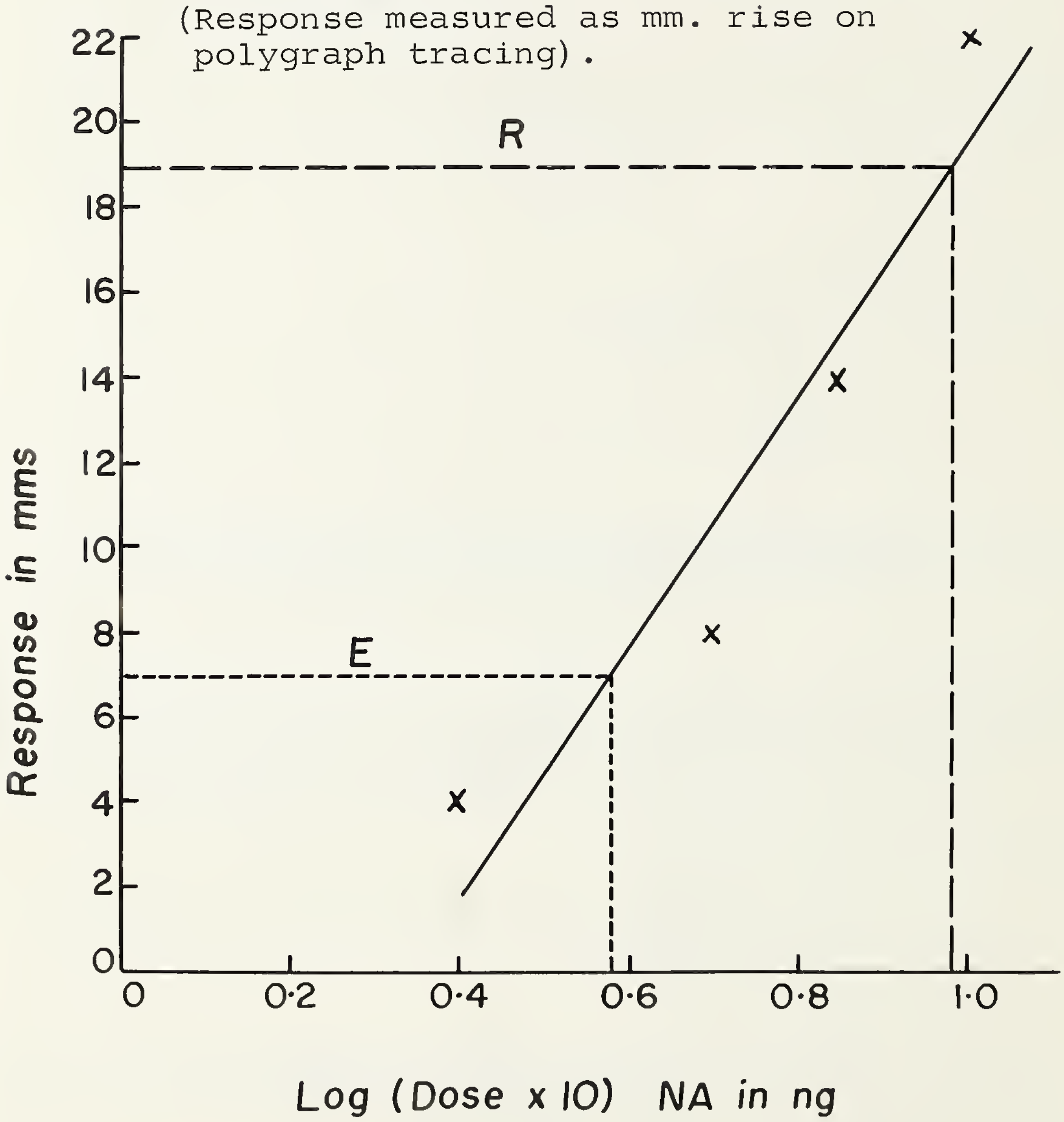
Therefore relative fluorescence per µg produced by A as compared with NA at 400 mµ = $\frac{A_{400}}{N_{400}} = \frac{15.21}{15.6} = 0.97$

Therefore 0.5 µg A = 0.5×0.97 µg NA as regards fluorimetric potency

" 0.5 µg A plus 0.5 µg NA = 0.99 µg NA

This system was used to compare the amounts of A and NA expressed

Log Dose-Response Line for Noradrenaline on Isolated Aorta
(Response measured as mm. rise on polygraph tracing).



as NA added, with the amounts of A and NA expressed as NA recovered.

BIOLOGICAL ESTIMATION OF CATECHOL AMINES IN AORTIC EXTRACTS

Calculation for Extract No. 3 (table 5)

R - internal standard (contained 200 ng NA before extraction)

E - extract from aorta of weight 58.5 mg., taken from an untreated rabbit.

Volume of acid eluate of E and R = 3.5 mls.

Amount added	Response	Equivalent log (dosex10) NA from graph	Equivalent log dose NA	Equivalent dose NA (antilog of log dose)
0.1ml.R	19 mm.	0.98	$\bar{1}.98$	1.0 ng
0.1ml.E	7 mm.	0.58	$\bar{1}.58$	0.38 ng

0.1 ml. R = 1.0 ng NA

But volume of R = 3.5 mls.

Therefore 3.5 mls. R contained $\frac{1.0 \times 3.5}{0.1}$ ng NA = total NA recovered

NA added = 200 ng

Therefore recovery = $\frac{1.0 \times 3.5 \times 100}{0.1 \times 200}$ = 17.5%

0.1 ml. E = 0.38 ng NA

But volume of E = 3.5 mls.

Therefore 3.5 mls. E contained $\frac{0.38 \times 3.5}{0.1}$ ng NA

Recovery = 17.5%

Therefore 3.5 mls. should contain $\frac{0.38 \times 3.5 \times 100}{0.1 \times 17.5}$ ng NA

= 76 ng NA

58.5 mg. aorta was extracted,

Therefore 1 gm. aorta contains $\frac{76 \times 1000}{58.5 \times 1000}$ μg NA

1.3 μg NA

0.69 μg NA base

APPENDIX III

EXPERIMENTS DESIGNED TO ASSESS THE SENSITIVITY AND ACCURACY OF THE EXTRACTION METHOD AND THE TRI-HYDROXYINDOLE METHOD FOR ASSAY OF ADRENALINE AND NORADRENALINE

METHODS

(1) Recovery Estimations Using Internal Standards

The whole thoracic aorta of a rabbit was removed and halved longitudinally. The tissue was divided longitudinally rather than transversely because there is evidence that the concentration of amines does not remain constant over the full length of the thoracic aorta.(40) The two pieces were equilibrated in Krebs-Henseleit as already described (page 15). A known amount of adrenaline or noradrenaline was added to one half, the other half being extracted and assayed without such an addition to obtain the concentration of amines in the aorta itself. Adrenaline or noradrenaline was added to the pulverised aorta dissolved in 1.0 ml. distilled water. In order to keep the extract volume constant at 2.0 mls., the concentration of perchloric acid was doubled and 1.0 ml. 0.6N added instead of 2.0 mls. 0.3N. The recovery of amines could be calculated by subtracting the concentration in the aorta from the concentration obtained using the piece of aorta to which adrenaline or noradrenaline had been added. The amines were calculated using the differential method and also using the method in which they are expressed as noradrenaline (Appendix II).

(2) Recovery Estimations Using Solutions of Adrenaline and Noradrenaline

As for the internal standards, known amounts of adrenaline and noradrenaline were added, dissolved in 1.0 ml. distilled water, to 1.0 ml. 0.6N perchloric acid and subjected to exactly the same extraction and assay procedure as the samples. Duplicate determinations of 1.0 μg adrenaline and 1.0 μg noradrenaline respectively, were carried

out. Mixtures of adrenaline and noradrenaline in decreasing concentration were assayed in order to determine the limits of sensitivity of the extraction and assay methods. All mixtures were assayed both differentially and expressed as noradrenaline (Appendix II).

(3) Differential Estimations of Adrenaline and Noradrenaline Solutions

Results from assays of samples, internal standards and solutions of adrenaline and noradrenaline taken through the extraction procedure, suggested that samples containing less than 0.1 μg adrenaline plus 0.1 μg noradrenaline in the final fluorescent solution, could not be assayed differentially. If, however, the amines were assayed and expressed as noradrenaline, values approximating those expected could be obtained. To test if this anomaly was due to the method of extraction or to the method of assay, a series of determinations using the tri-hydroxyindole fluorimetric technique on solutions of adrenaline and noradrenaline of known concentration was carried out. These assays also served to indicate the sensitivity and accuracy of the assay technique.

(a) Adrenaline and noradrenaline were assayed separately in solutions of varying concentration.

(b) Adrenaline and noradrenaline were assayed in mixtures, containing equal amounts of adrenaline and noradrenaline, in concentrations of 0.1 μg/ml. (final fluorescent solution) and less.

(c) Adrenaline and noradrenaline were assayed in mixtures, containing different relative proportions of adrenaline and noradrenaline, in concentrations of 0.1 μg/ml. (final fluorescent solution) and less.

TABLE 9

DIFFERENTIAL ESTIMATION OF ADRENALINE AND/OR NORADRENALINE

(A and NA expressed in μg and as the total amount of added)

No.	A added	A recovered	NA added	NA recovered	A+NA as NA added	A+NA as NA recovered
1	0.1	0.08	-	-	-	-
2	0.01	0.005	-	-	-	-
3	-	-	0.1	0.07	-	-
4	-	-	0.01	0.006	-	-
5	1.0	0.93	1.0	0.94	1.96	1.83
6	0.1	0.1	0.1	0.09	0.2	0.18
7	0.01	0.02	0.01	0.006	0.02	0.02
8	1.0	0.81	1.0	1.01	1.97	1.8
9	1.0	0.88	0.5	0.49	1.48	1.36
10	1.0	0.85	0.1	0.12	1.07	0.95
11	1.0	0.84	0.01	0.1	0.98	0.91
12	0.5	0.42	1.0	0.82	1.49	1.23
13	0.1	0.08	1.0	0.75	1.1	0.84
14	0.01	-ve	1.0	0.65	1.01	0.63

(Explanation of negative results is given in Appendix II)

TABLE 8

RECOVERIES OF ADRENALINE AND/OR NORADRENALINE TAKEN THROUGH THE EXTRACTION AND ASSAY PROCEDURE

(A and NA expressed in μg and as the total amount added)

Expt. No.	A Added	A recovered	NA added	NA recovered	A+NA added as NA	A+NA recovered as NA	Mean percent recovery
1	1.0	0.97	-	-	-	-	94.5%
2	1.0	0.92	-	-	-	-	
3	-	-	1.0	0.78	-	-	78%
4	-	-	1.0	0.78	-	-	
5	0.5	0.48	0.5	0.4	0.99	0.83	82%
6	0.5	0.49	0.5	0.27	0.99	0.74	
7	0.5	0.38	0.5	0.51	1.02	0.9	
8	0.1	0.16	0.1	0.05	0.2	0.21	122%
9	0.1	0.16	0.1	0.09	0.2	0.25	
10	0.1	0.12	0.1	0.14	0.2	0.27	
11	0.01	0.04	0.01	0.03	0.02	0.07	350%
12	0.01	0.04	0.01	0.04	0.02	0.08	
13	0.01	0.05	0.01	0	0.02	0.06	

The differential method and the method in which the amines are expressed as noradrenaline were used in the calculations.

DISCUSSION OF RESULTS

Amounts of adrenaline and noradrenaline quoted are expressed as the amount of amine in 10 mls. fluorescent solution.

From table 8 it is evident that in samples containing less than 0.5 μg noradrenaline plus 0.5 μg adrenaline, the extraction method combined with the differential assay did not yield accurate results. If, however, the amines were assayed and expressed as noradrenaline, estimates of samples containing 0.1 μg noradrenaline plus 0.1 μg adrenaline could be obtained with an average error of 22%. Samples containing 0.01 μg noradrenaline plus 0.01 μg adrenaline could not be estimated with any degree of accuracy, even by assaying and expressing the total amines as noradrenaline.

Assaying and expressing the amines as noradrenaline:

Mean recovery when 0.5 μg of each amine was added = 82%
" " " 0.1 μg " " " " " = 122%
" " " 0.01 μg " " " " " = 350%

Table 9 demonstrates the degree of accuracy and sensitivity of the assay method itself. Mixtures of adrenaline and noradrenaline, whether in equal or different amounts, could be assayed differentially with an error of not more than 25%, so long as the amount of either amine was not less than 0.1 μg. The error in estimating differentially mixtures containing 0.01 μg of either amine varied between 40% and 100%. However, by assaying and expressing the amines as noradrenaline, the error

TABLE 10

RECOVERIES OF ADRENALINE AND NORADRENALINE ADDED TO AORTA AND TAKEN THROUGH THE EXTRACTION AND ASSAY PROCEDURES

Expt. No.	A added to aorta	A recovered	NA added to aorta	NA recovered
1	1 μg	0.64 μg		
2	1 μg	0.78 μg		
3			1 μg	0.65 μg
4			1 μg	0.65 μg

was reduced thus:

Composition of mixture of A and NA	Error of A plus NA assayed as NA
1.0 μg A+0.01 μg NA	7.3%
0.01 μg A+1.0 μg NA	34%
0.01 μg A+0.01 μg NA	0%

It can be deduced by comparing tables 8 and 9 that the accuracy of the assay is reduced by the extraction procedure. This is probably due to loss of the amines during extraction (table 10). For instance, samples of 0.1μg adrenaline plus 0.1 μg noradrenaline cannot be accurately extracted and assayed differentially but this mixture can be accurately assayed differentially if there is no extraction. Similarly, mixtures of 0.01 μg adrenaline plus 0.01 μg noradrenaline, assayed and expressed as noradrenaline, had an error of over 200% if the extraction procedure was used before assay.

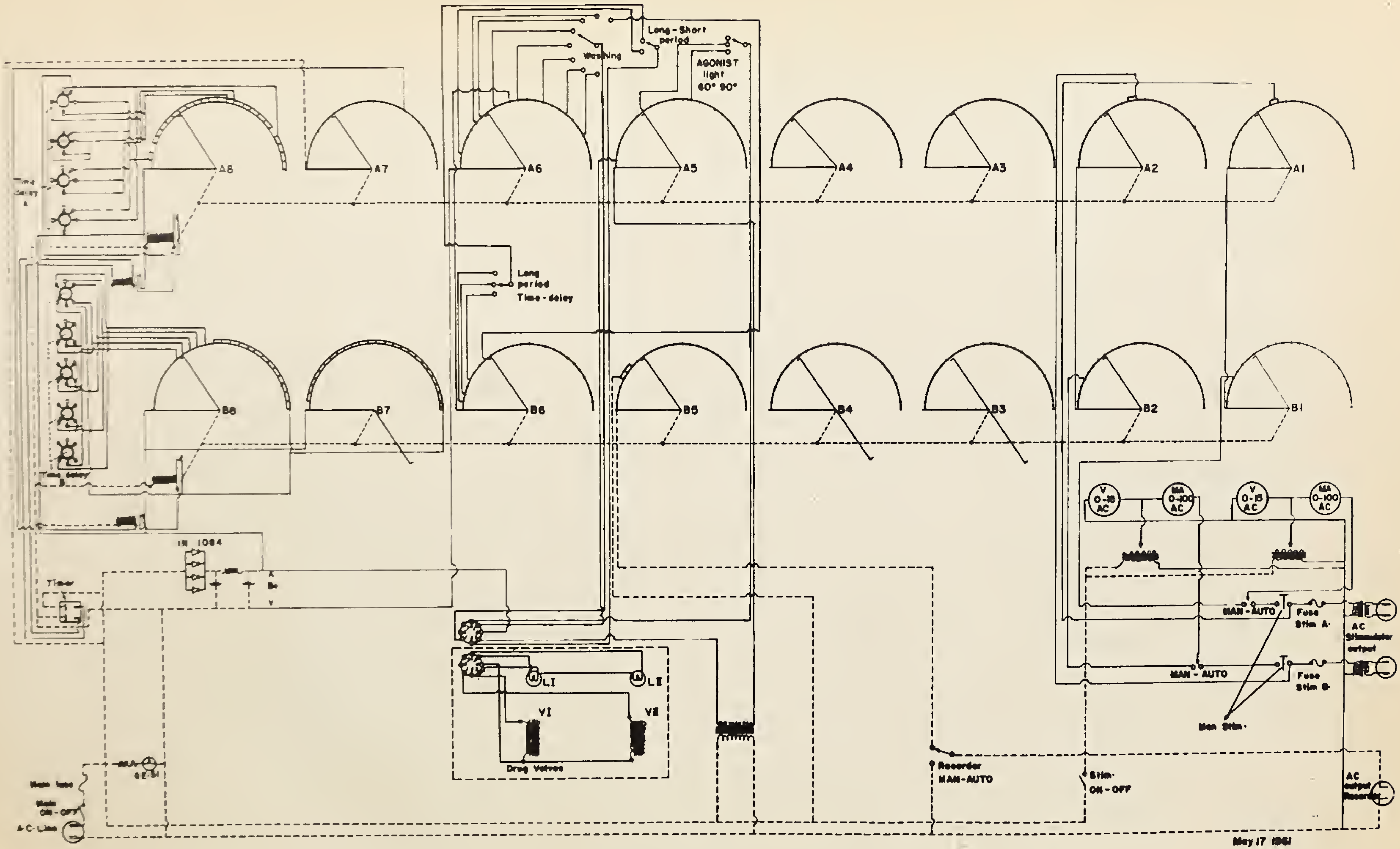
Therefore, with the methods of extraction and assay described, the lower limit for assay of catechol amines as noradrenaline lies between 0.2 μg and 0.02 μg catechol amines per sample.

APPENDIX IV

AN ELECTRICAL MECHANISM USED TO STIMULATE TISSUE ELECTRICALLY AT REGULAR INTERVALS

Figure XVII·

Circuit Diagram·



May 17 1961
Ralph Hollenberg

The circuit diagram (figure XVII) is of a very versatile electrical mechanism, based on a design by Gaddum and Lembeck(109), which can be used to operate an organ bath automatically. The only part of this mechanism which was used in the work described in this thesis, was that concerned with the automatic electrical stimulation of a piece of isolated vascular tissue. A brief description of the latter is therefore given.

Two stepping relays A and B, each relay having 8 banks and each bank 25 contacts over 180°, were used to produce the time delays. Since relay A received a pulse every 5 seconds from a time clock, the time for the rotating lever on A to describe 180° equalled 5 x 25 or 125 seconds. Once every cycle bank A7 activated relay B through bank B8. Thus the total time cycle afforded by the combination of relay A and relay B was 25 x 125 or 3125 seconds. By appropriate shorting of contacts on relay(s) A and/or B it was possible to reduce this time cycle as desired. A time cycle of 12.5 minutes, which was suitable for the electrical stimulation of aorta, could thus be obtained.

The A.C. stimulus was initiated every 12.5 minutes by bank B1. Bank A1 was responsible for fixing the duration of stimulation at 10 seconds. Banks B2 and A2 performed exactly the same functions for a second stimulator. The stimulus was fed into the tissue through an isolating transformer.

The recorder was switched on, 1 minute 40 seconds before the stimulus occurred, by activation of the appropriate contacts on bank B5. After the 10 second stimulus, the recorder remained on for a further 4 minutes 25 seconds.

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